THE STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF THE MAJOR OUTER MEMBRANE PROTEIN OF *CHLAMYDIA PSITTACI*

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DECLARATION

The work reported in this thesis was carried out under the supervision of Dr. Alan J. Herring and Dr. David Longbottom at the Moredun Research Institute and Dr. Richard H. Ashley at the Biochemistry Department, University of Edinburgh. All results presented, unless otherwise stated, are the sole work of the author, as is the composition of this thesis.

To Mum and Dad

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ABBREVIATIONS

A	Amperes
ATP	Adenosine 5′- triphosphate
bp	Base pair(s)
BSA	Bovine serum albumin
cAMP	Adenosine 3′, 5′ - cyclic monophosphate
CD	Circular dichroism
cDNA	complementary DNA
CIAP	Calf intestinal alkaline phosphatase
COMC	Chlamydial outer membrane complex
CRPs	Cysteine rich proteins
dNTPs	Deoxynucleoside 5′-triphosphates
Da	Dalton
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EB	Elementary body
EDTA	Ethylenediaminetetra-acetic acid
F	Faraday constant
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GdmCl	Guanidinium chloride
GHK	Goldman Hodgkin Katz equation

h	Hours
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethane-sulphonic acid
hsp	Heat shock protein
IFU	Inclusion forming units
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL	Interleukin
Inc	Inclusion membrane protein
IPTG	Isopropyl-β-D-thiogalactoside pyranoside
J	Joule
Kdo	Keto-3-deoxy-D-manno-octulosonic acid
LGV	Lymphogranuloma venereum
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
MIF	Micro-immunofluorescence
min	Minute
MIP	Macrophage infectivity potentiator
MOMP	Major outer membrane protein
mRNA	Messenger ribonucleic acid
NBS	Neonatal bovine serum
NMR	Nuclear magnetic resonance
OEA	Ovine enzootic abortion
OG	n-Octyl-β-D glucopyranoside

Omp	Outer membrane protein
ORF	Open reading frame
Octyl POE	Octyl polyoxoethylene
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS containing Tween 20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pI	Isoelectric point
PID	Pelvic inflammatory disease
PMSF	Phenylmethylsulfonyl fluoride
POMPs	Putative outer membrane proteins
R	Gas constant
RB	Reticulate body
RE	Restriction endonuclease
RFLP	Restriction fragment length polymorphism
S	Siemens
s	Seconds
SDS	Sodium dodecyl sulphate
Sarkosyl	N-Lauroyl-sarcosine
STD	Sexually transmitted disease
TAE	Tris-acetate containing EDTA
TE	Tris containing EDTA
TES	TE containing sucrose

TESL	TES containing lysozyme
Th	T helper cell
TID	Trifluoro-methyl-3-iodophenyl-diazirine
tMOMP	Truncated MOMP
Tris	Tris [hydroxymethyl] aminomethane
TSS	Transport and storage solution
TWAR	Taiwan acute respiratory strain
UV	Ultraviolet
V	Volt
VS	Variable segments
v/v	Volume/volume
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-thiogalactopyranoside
z	Valency

ABSTRACT

The major outer membrane protein (MOMP) of *Chlamydia* shares several biochemical properties with classical porin proteins. Secondary structure analysis by circular dichroism now reveals that MOMP from *Chlamydia psittaci*, purified by hydroxyapatite chromatography, has a predominantly β-sheet content (62%), typical of bacterial porins. To directly test the "porin channel" hypothesis at the molecular level, MOMP was reconstituted into planar lipid bilayers where it gave rise to "triple-barrelled" channels which were modified by an anti-MOMP neutralising monoclonal antibody. These observations are consistent with the well characterised homo-oligomeric nature of MOMP previously revealed by biochemical analysis, and the "triple-barrelled" behaviour of other porins. MOMP channels were weakly anion selective ($P_{Cl}/P_K \sim 2$) and permeable to ATP. They may therefore be a route by which *Chlamydia* can take advantage of host nucleoside triphosphates, and explain why some anti-MOMP antibodies neutralise infection.

In order to undertake more detailed studies of the MOMP structure/function relationship, recombinant MOMP from both *C. psittaci* and *C. pneumoniae* have been cloned and expressed. The recombinant proteins were functionally reconstituted in planar lipid and analysed at the single channel level. Both form porin-like ion channels that are functionally similar to the native protein. The *C. psittaci* recombinant porin was modified by the same anti-MOMP neutralising monoclonal antibody that affected the native protein. In contrast to the native protein, both recombinant *C. psittaci* ($P_{Cl}/P_K \sim 0.38$) and *C. pneumoniae* ($P_{Cl}/P_K \sim 0.49$) proteins were marginally cation selective. This is the first time native function has been demonstrated for recombinant chlamydial MOMP and will have an important impact on the future development of subunit vaccines.

The method by which chlamydiae attach to and infect eukaryotic cells is unclear. It has been suggested that *Chlamydia* synthesise a heparan-sulphate-like glycosaminoglycan which acts as a bridge by binding to receptors on both the eukaryotic cell and chlamydiae. The addition of an exogenous source of heparin resulted in a dose-dependent decrease in the levels of infection of the OEA isolate of *C. psittaci* as did pre-treatment of chlamydiae with heparan-sulphate lyase. Radioactively labelled heparin was found to bind specifically to Western blots of native MOMP, suggesting that MOMP may also function as the chlamydial GAG receptor.

CHAPTER ONE

INTRODUCTION AND AIMS

1.1 *Chlamydiales* and their taxonomy

Chlamydia have been assigned to their own order *Chlamydiales* (Storz and Page, 1971) within which there is one family *Chlamydiaceae*, comprising a single genus *Chlamydia* and, at present, four species. The classification of *Chlamydia* in their own order reflects their unique obligate intracellular, biphasic lifecycle which alternates between the infectious, metabolically inert elementary body (EB) and the non-infectious, metabolically active reticulate body (RB). In the past, the unusual properties of chlamydiae has lead to their mis-classification. In 1907 Halberstaedter and von Prowazek (1907) isolated what they determined to be a protozoan from the conjunctival scrapings of an infected patient and called this parasite chlamydazoon. Subsequently, as they could not be grown by normal plating techniques, chlamydiae were identified as viruses and this theory continued until 1966 when Moulder finally identified them as intracellular gram negative bacteria (Moulder 1966).

Sarov and Becker (1968) were the first to discover that *Chlamydia* had all the requisite properties of bacteria. Indeed, Weisburg *et al.* (1986) found that comparison of the 16S rRNA gene sequences of *C. psittaci* with those of other organisms indicated that *Chlamydia* are eubacterial in origin. Phylogenetic analysis of these sequences revealed that the *Chlamydiales* may be peripherally related to the Planctomyces. However, the link may be tenuous as the only phenotypic characteristics the two groups are known to have in common is a cell wall without detectable peptidoglycan (Section 1.5.1) (Liesack *et al.,* 1986).

Until recently, only two species of *Chlamydia* were recognised, *Chlamydia trachomatis* and *Chlamydia psittaci*, assigned to the genus on the basis of differences in: (1) inclusion body morphology (2) glycogen content (3)

susceptibility to sulfadiazine and (4) natural hosts. *C. trachomatis* is primarily a human pathogen. In addition to causing blinding trachoma in arid regions, *C. trachomatis* infections of the genital tract are now recognised as a major cause of pelvic inflammatory disease, often resulting in infertility (Batteiger and Jones, 1987). *C. psittaci* is largely a pathogen of animals. However, avian strains of *C. psittaci* are still an occasional cause of fatal human infection and are a widespread problem in domestic turkeys, ducks and pet birds. Mammalian strains of *C. psittaci* are a major cause of abortion in ruminants and can also cause abortion in women infected during pregnancy (Section 1.2.3) (Buxton 1986).

These two species of *Chlamydia* share only 10% DNA homology as shown by DNA hybridisation experiments and are easily differentiated on the basis of restriction endonuclease analysis (Kingsbury and Weiss, 1968; Weiss *et al.,* 1970). The species *C. trachomatis* is further divided into 3 biovars, mouse, lymphogranuloma venereum (LGV) and trachoma. The trachoma biovar currently consists of 19 serovariants (serovars). The LGV biovar is divided into 4 serovars L1, L2, L2a and L3. The classification of the highly diverse *C. psittaci* strain has been based on a number of biological, immunological and genetic criteria. Perez-Martinez and Storz (1985) proposed 9 immunotypes of mammalian *C. psittaci* strains while Tayofuku *et al.* (1986) indicated the presence of 7 avian strains. *C. psittaci* isolated from ruminants were grouped into immunotype 1 (abortion and some intestinal types), immunotype 2 (polyarthritus, conjunctivitis, encephalitis and enteritis types) and immunotypes 3 and 9 (types isolated from bovine and ovine faeces) according to the system of Perez-Martinez and Storz.

The realisation that the novel *Chlamydia* agent, designated TWAR (Taiwan acute respiratory strain), was an important human respiratory pathogen drew

attention to its mis-classification within the *C. psittaci* species (Grayston *et al.,* 1986, 1989). With respect to the criteria used to differentiate species of *Chlamydia*, TWAR was more closely related to *C. psittaci* than *C. trachomatis*. However, DNA analysis revealed less than 10% relatedness between TWAR and other *C. psittaci* strains (Cox *et al.,* 1988). Homology among TWAR strains is 94% or greater. These results prompted the establishment of a new third species of *Chlamydia*, called *Chlamydia pneumoniae* (Grayston *et al.,* 1989).

The genus *Chlamydia* has recently been further expanded to accommodate a new species of ruminant strains proposed by Fukushi and Hirai (1992) and called *Chlamydia pecorum.* It has long been established that at least two types of *Chlamydia* infect ruminants, the immunotype 1 strains associated with abortions and occasionally found in faeces, and other strains which are associated with polyarthritis, polyserositis, encephalitis and inapparent enteric infections. Studies of a subset of 5 strains of this latter type have found them to be genetically and antigenically distinct from all other *Chlamydia* species and to form a new fourth species, *C. pecorum* (Fukushi and Hirai, 1988, 1989).

PCR-based methods have substantially advanced the typing of *Chlamydia.* The major outer membrane protein (MOMP) gene (Section 1.8), the site of most strain and species antigenic variation, provides an ideal target for PCR as it has highly conserved regions at both ends flanking variable regions. Numerous typing protocols have involved the amplification of the coding regions of the MOMP gene followed by RE analysis or sequencing of the amplified product (Kaltenboeck *et al.,* 1991; Rodriguez *et al.,* 1991; Frost *et al.,* 1991). This combination of typing techniques has proved highly effective. However, the recent sequencing of the 16S and 23S intergenic regions of chlamydial ribosomal DNA has provided an

alternative, rapid and reproducible method for the identification and classification of chlamydial strains (Everett and Andersen, 1997). Indeed, phylogenetic analysis of data obtained using this technique have called into question the appropriateness of the current classification of *Chlamydia*.

1.2 Chlamydial disease

1.2.1 *Chlamydia trachomatis*

Members of the genus *Chlamydia* are important pathogens of humans, birds and other animals. Indeed, *Chlamydia* are one of the most ubiquitous pathogens in the animal kingdom. *C. trachomatis*, the best studied of the four chlamydial species, is a significant agent of sexually-transmitted and ocular infections in humans (Schachter *et al.,* 1975). Trachoma is the leading cause of preventable blindness in the world with over six million blind as a result of the disease. The onset of the disease is often inconspicuous, appearing as a mucopurulent conjunctivitis. Over time, scarring of the conjunctivae develops and the cornea may also be affected by the formation of a pannus (vascularization of the corneal limbus). The contraction of the scars within the conjunctiva distort the eyelid and cause an in-turning of the eyelashes so that they abrade the cornea. Severe lid deformity may not develop until 20 or 30 years after infection. Subsequent bacterial infections lead to the progressive deterioration of the diseased eye. Treatment of trachoma is currently based on the topical application of tetracycline ointments.

In industrialised countries *C. trachomatis* is the most prevalent cause of sexually transmitted disease (Schachter *et al.,* 1975) resulting in the less serious conditions of urethritis, cervicitis, and the more serious infections epididymitis,

ectopic pregnancy and pelvic inflammatory disease (PID). Chlamydial infections in the male urethra result in a broad range of symptoms, from infections producing a discharge to those which are asymptomatic. However, chlamydial urethritis is usually seen as a mucopurulent discharge in a man suffering from dysuria and has an incubation period of 1-3 weeks (Oriel and Ridgeway, 1982). While chlamydial urethritis superficially appears to be a trivial infection, untreated it can result in severe complications. Ascending urethritis can occur, resulting in epididymitis (Berger *et al.,* 1978).

The cervix is the area of female genitalia most commonly affected by chlamydial infection, where the organism can result in cervicitis (Paavonen *et al.* 1982; Storz and Krauss, 1983). Again, infection is not always accompanied by easily diagnosed symptoms, however, discharge is common. Ascending *C. trachomatis* infection is also common in females, usually targeting the endometrium or fallopian tubes (Mardh *et al.,* 1977). The resulting damage and scarring from these acute infections can lead to tubal factor infertility and ectopic pregnancy (Cates, 1984). Uncomplicated genital tract conditions can be treated with tetracycline, however, more chronic infections require long term therapy.

1.2.2 *Chlamydia pneumoniae*

C. pneumoniae, a recent addition to the genus *Chlamydia*, has been shown to cause community acquired and nosocomial pneumonias and to be a common infectious agent throughout the world (Kauppinen and Saikku, 1995; Lieberman *et al.,* 1996). An association between *C. pneumoniae* and vascular atheroma and ischaemic heart disease resulting in acute myocardial infarction was established using the classical micro-immunofluorescence test (MIF) (Saikku *et al.,* 1988) and

has been verified seroepidemiologically in over 20 studies (Danesh *et al.,* 1997). Data presented at the 9th International Symposium on Human Chlamydial Infection, Napa, California, USA appears to confirm a causal role for *C. pneumoniae* in the development of atherosclerotic disease. Using a transgenic mouse model deficient in apolipoprotein, Moazed *et al.* (1998) reported that *C. pneumoniae* infection resulted in an 136% increase in atherosclerotic lesion size, compared with uninfected controls, at 16 weeks postinfection. However, difference in lesion size at 20 weeks was not so marked suggesting that *C. pneumoniae* infection accelerates the atherosclerotic process in the early to intermediate stages of lesion development.

There is little evidence to date describing how *C. pneumoniae* initiates or contributes to the development of atherosclerosis. Atheroma initiation and development is thought to involve macrophage foam cells, subendothelial macrophages which have ingested cholesterol. Kalayoglu and Byrne (1998) have reported that human monocyte-derived macrophages infected with *C. pneumoniae*, accumulate neutral lipids and cholesteryl esters thereby developing into foam cells, suggesting a possible causal role for *C. pneumoniae* in atherosclerosis.

1.2.3 *Chlamydia psittaci*

C. psittaci commonly infects a wide variety of mammals and birds and has been implicated in a range of disease conditions. One of the best studied and most economically important *C. psittaci*-caused diseases is ovine enzootic abortion (OEA) (Aitken *et al.,* 1990). Indeed, placental and fetal infections with chlamydiae are a significant cause of reproductive failure and abortion in cattle, goats and many other domestic animals, in addition to sheep (Storz and Krauss, 1985). The ovine abortion strains of *C. psittaci*, infecting pregnant ewes, result in a necrotizing

placentitis and consequent abortion, usually within the final month of gestation. Bovine *Chlamydia*-induced abortions tend to occur in the last trimester. Pathological examination of an infected placenta post partem reveals the appearance of placental necrotic lesions, the margins of which consist of zones of hyperemia and haemorrhage. Contact with *C. psittaci*-infected sheep during pregnancy can lead to the abortion of a human fetus (Buxton 1986).

It has been demonstrated that *C. psittaci*-infection can result in ocular disease in several animal species. Severe conjunctivitis is observed in many species and is often associated with a more serious secondary chlamydial infection. This is exemplified by calves which have systemic chlamydial infections, leading to polyarthritis, also developing conjunctivitis. In addition, Stephenson *et al.* (1974) noted that virtually all lambs diagnosed with polyarthritis also had conjunctivitis. The same strain of *Chlamydia* was cultured from the conjunctiva and joint samples of these lambs.

Avian chlamydiosis is a major public health risk and results in large economic losses in the poultry industry. The most common route of chlamydial infection for birds is via the respiratory tract. Transmission occurs predominantly by inhalation of infected dust. Infected birds shed the organism within their faeces and the dried excrement remains infectious for several months (Page 1959). The acute disease in cage birds is characterised by diarrhoea and anorexia. These symptoms can be accompanied by respiratory distress and conjunctivitis.

1.2.4 *Chlamydia pecorum*

C. pecorum, until recently classified as a subtype of *C. psittaci*, has several interesting properties. It possesses a broad host range from bovine to swine, even

koala. Associated diseases range from severe encephalitis, pneumonia, and enteritis in cattle and infectious polyarthritis in sheep. In addition, one subtype of *C. pecorum* has been implicated in bovine metritis resulting in hypofertility (Wittenbrink *et al.,* 1993).

1.3 Pathogenesis

Chlamydial infections are characterised by intense and persistent local inflammation. The host response to primary infection at mucosal surfaces occurs within 1-2 days and is characterised by inflammation and mucosal infiltration with neutrophils and small numbers of monocytes. In addition, T-cells accumulate at the site of infection and play a crucial role in controlling infection. Recently, Rasmussen *et al.* (1998) demonstrated that *Chlamydia*-infected epithelial cells express and secrete increased levels of chemokines IL-8 and GRO-α, in addition to cytokines GM-CSF, IL-1α and IL-6. Several of these cytokines are potent chemoattractants and activators of neutrophils, monocytes and T-lymphocytes. It is proposed that they stimulate the recruitment of the inflammatory cells to the site of infection. In most cases the response to primary infection is transient, however, a prolonged inflammatory response may well contribute to fibrosis and tissue scarring, characteristic of chronic ocular and genital *C. trachomatis* disease.

It has long been established that chlamydial infection can illicit delayed-type hypersensitivity (DTH). Grayston *et al.* (1985) presented strong evidence to suggest re-infection is required and enhances disease. Over a 10 year period they monitored 32 families with incidence of *C. trachomatis* infection. The most severe and progressive trachoma infections, with panus and scar formation, occurred only after

re-infection (Grayston *et al.,* 1985). Previous studies using animal models showed that repeated ocular exposure to infectious chlamydiae is necessary to establish the chronic inflammation characteristic of trachoma (Wang and Grayston, 1962; Wang and Grayston, 1967; Taylor *et al.,* 1982). Interestingly, repeated challenge with infectious chlamydiae produces a sub-mucosal cellular infiltrate of lymphocytes and macrophages, like that observed in individuals with trachoma. Collectively, both human and animal studies argue for a pathogenic role of DTH in disease.

A direct mechanistic link between DTH and disease has not been established. Replication of the organism in the host is not required to illicit the heightened tissue reaction suggesting that a constitutive antigen found in chlamydiae and not unique to actively growing chlamydiae is sufficient to trigger the deleterious immune response (Taylor *et al.,* 1981; Taylor *et al.,* 1982; Watkins *et al.,* 1986). As heterologous strains of *C. trachomatis* prime the immune system for an immune response upon re-infection, the hyper-sensitive antigen is most likely cross-reactive and shared among different chlamydial strains. A wide range of research suggests that a single 57 kDa protein is centrally involved in pathogenesis. Brunham *et al.* (1985) observed that 11 out of 13 women with *C. trachomatis*-associated tubal infertility had serum antibodies to a 57 kDa chlamydial antigen, compared to 1/11 healthy *C. trachomatis* seropositive control women. Subsequently, the gene for this 57 kDa protein was cloned, sequenced (Cerrone *et al.,* 1991) and evaluated in animal models of disease pathogenesis (Morrison *et al.,* 1989). The protein was identified as a chlamydial GroEL homologue (member of the hsp60 family) that is common to the *Chlamydia* genus (Bavoil *et al.,* 1990). Purified recombinant hsp60 protein was a potent T-cell antigen and elicited an intense inflammatory response when applied to the mucosal surfaces of the fallopian

tube of prior immunised animals (Patton *et al.,* 1994).

The actual mechanism by which hsp60 can illicit a hyper-immune response has not been determined, however, several hypotheses have been proposed (Brunham and Peeling, 1994). Hsp60 is a highly conserved protein, with the human and chlamydial sequences sharing 48% identity at the amino acid level (Cerrone *et al.,* 1991). The recognition of hsp60 by the host immune system may incite an autoimmune inflammatory reaction. Alternatively, high level antibody responses to chlamydial hsp60 may signal chronic persistent or repeated chlamydial infection with other chlamydial antigens sustaining the chronic inflammatory reaction.

There is evidence to suggest that genetic pre-disposition may play some part in the development of scarring sequelae after chlamydial infection. Holland *et al.* (1996) proposed that individuals who develop conjunctival scarring, after infection with *C. trachomatis*, lack a Th1 cell-mediated immune response and cannot clear the infection. Therefore individuals developing a chronic infection would presumably have a strong Th2 response with high antibody titres to the organism and weak Th1 responses. This hypothesis is based on observations that patients with conjunctival scarring appear to have reduced proliferative responses to *C. trachomatis* EBs.

In conclusion, both clinical and experimental data indicate that disease pathogenesis is due to immunologically mediated tissue injury. Immune responses to the chlamydial hsp60 correlate with the severity of the disease and long term damage. However, the precise nature of the immune-mediated tissue damage and relationship to hsp60 is unclear.

1.4 Chlamydial lifecycle

1.4.1 Adhesion and internalisation

The initial events in chlamydial pathogenesis, attachment of the infectious EB to a susceptible cell followed by internalisation, are poorly understood and studies concerning these processes have produced conflicting results. However, it would seem reasonable to assume that the surface components which must play an important role in these early steps of chlamydial infection are key virulence determinants. Their identification and molecular characterisation are crucial to our understanding of chlamydial pathogenesis and the development of anti-chlamydial strategies.

Chlamydiae infect columnar epithelial cell of mucous membranes, while serotypes L1-3 of *C. trachomatis* also proliferate in lymphatic tissue causing systemic infection. The binding of EBs to host cells obeys saturation kinetics suggesting the involvement of a specific ligand-receptor interaction (Byrne 1978). The quest to identify chlamydial adhesins has focused on the roles of chlamydial proteins. The involvement of a proteinaceous component was demonstrated by Joseph and Bose (1991) who found that both heat and trypsin treated EBs failed to competitively inhibit the adherence of untreated EBs in an *in vitro* binding assay.

Many candidates have been implicated as the proteinaceous component mediating the attachment of EBs to target cells. The MOMP is an obvious candidate as a potential adhesin because of its predominance in the outer membrane. MOMPs role in adherence is based primarily on the inhibitory properties of trypsin and monoclonal antibodies which target its surface exposed variable domains. Su *et al.* (1990) demonstrated that trypsin proteolysis of the MOMP, cleaving sites in variable segments II and IV (Section 1.8.3) resulted in a significant loss of

attachment for two serovars of *C. trachomatis*. Monoclonal antibodies specific to antigenic determinants located on these segments neutralise the infectivity of *C. trachomatis* serovar B by blocking adhesion (Collett *et al.,* 1989). It has been proposed that the MOMP functions as a chlamydial adhesin by promoting non-specific (electrostatic and hydrophobic) interactions and that the binding of monoclonal antibodies to the MOMP blocks attachment by inhibiting the electrostatic interactions with the host cell (Hatch *et al.,* 1981). Other proteins implicated in the attachment process include a 38 kDa chlamydial protein shown to be surface exposed by radio-iodination and found to be thermolabile and trypsin sensitive (Joseph and Bose, 1991). This 38 kDa protein was found to bind specifically to glutaraldehyde-fixed HeLa cells and binding was competitively inhibited by whole EBs, indicating a shared receptor. In addition, the cysteine rich Omp2 (Section 1.5.1) has also been suggested as a possible cytadhesin despite the ambiguity over its location in the chlamydial envelope (Ting *et al.,* 1995). The participation of glycoproteins in the mechanism of attachment to eukaryotic cells has also been proposed due to inhibition in chlamydial binding assays upon the addition of the lectin wheat germ agglutinin.

Recent studies have suggested that the MOMP of *C. trachomatis* may be glycosylated (Section 1.8.1) (Swanson and Kuo, 1991). The [^{3}H] glycan was shown to attach readily to HeLa cells and binding was found to be competitive with whole EBs indicating a role in the attachment process (Kuo *et al.,* 1996).

Perhaps the most convincing hypothesis for chlamydial attachment to eukaryotic cells is that recently proposed by Zhang and Stephens (1992). This involves the synthesis of a molecular mimic of heparan-sulphate by *Chlamydia*, which can bind to the host cell exploiting receptors used for eukaryotic cell-cell

interactions. This glycosaminoglycan (GAG) is thought to mediate attachment by bridging mutual GAG receptors on the host cell surface and on the chlamydial outer membrane surface. Attachment of radio-labelled *C. trachomatis* (Chen and Stephens, 1997) and fluorescently labelled *C. psittaci* (Gutierrez-Martin *et al.,* 1997) were inhibited by the presence of heparin or heparan sulphate, however, low concentrations of heparan sulphate potentiated attachment. Treatment of chlamydiae with heparan sulphate lyase was found to ablate infectivity, whereas the addition of exogenous heparan sulphate restored their ability to attach and infect (Gutierrez-Martin *et al.,* 1997; Chen and Stephens, 1997). Despite this convincing evidence it is not suggested that this GAG mediated pathway is the only mode of attachment and infection exploited by *Chlamydia.* Indeed, differences have been observed between *C. trachomatis* biovars in the extent to which heparin effects their adherence and infectivity. The LGV biovar appears to predominantly use a heparin-inhibitable mechanism for attaching to host cells, whereas the trachoma biovar uses both heparin-dependent and independent mechanisms to adhere and a heparin-dependent pathway to enter host cells.

The chlamydial outer membrane proteins that bind heparan sulphate are unknown. Possible candidates include MOMP and the 60 kDa protein. A MOMP fusion protein which is known to bind specifically to epithelial cells has been shown to be inhibitable by the addition of heparan sulphate or by the heparitinase treatment of the cells (Su *et al.,* 1996). Furthermore, the attachment of the MOMP fusion protein was markedly reduced to mutant cell lines defective in heparan sulphate synthesis. These results strongly suggest that the MOMP is the chlamydial GAG receptor.

Studies concerning the process of entry into host cells have produced conflicting results, and most workers believe that *Chlamydia* utilise more than one mode of entry. Proposed mechanisms of uptake include parasite-specified phagocytosis, receptor-mediated endocytosis in clathrin coated pits and pinocytosis in non-coated pits. The most convincing *in vitro* approach used to address this question was the use of hormonally maintained primary cultures of human endometrial gland epithelial cells in polarised culture. Applying this technique, Wyrick and co-workers (Wyrick *et al.,* 1993) were able to visualise *C. trachomatis* in coated vesicles and pits using transmission electron microscopy indicating that there is a role for the clathrin facilitated uptake of chlamydiae. This observation supports the earlier work of Soderlund and Kihlstrom (1988) who found that primary amines, inhibitors of host cell receptor recycling, interfere with the internalisation of chlamydiae, suggesting that coated pit pinocytosis may be involved. However, Ward and Murray (1984) failed to see chlamydiae associated with coated pits over a 30 minute period, but did observe a reduction in entry of chlamydiae following treatment of host cells with cytochalasin D, an inhibitor of microfilament-dependent phagocytosis. Pearce and Reynolds (1991) obtained data suggesting that at least two pinocytic entry mechanisms are exploited by chlamydiae. They proposed that chlamydiae can enter through a pathway involving coated pits and through an inducible pathway independent of coated pit uptake. Therefore, it would seem that chlamydiae can utilise several entry mechanisms enabling them to infect a wide variety of cell types in a variety of conditions.

1.4.2 EB to RB transition

Regardless of mode of entry, EBs are found to exist intracellularly within vesicles derived from the host cell plasma membrane. Whereas *C. trachomatis* containing vesicles fuse together shortly after entry to form one large vesicle, termed the inclusion, *C. psittaci*-containing vesicles remain separate from one another resulting in multiple inclusions (Gordon and Quan, 1965). At 2-12 hours post-infection, the EB transforms into the replicative, metabolic+ally active RB. As a result of this transformation, the EB loses its prominent electron-dense DNA core, the cell envelope becomes less rigid, its size increases from approximately 0.3 to 1.0 μm in diameter and its cytoplasm becomes granular due to the production of ribosomes. The RB has an osmotically fragile cell wall resulting from the reduction of the disulphide bond cross-linking of the proteins in the chlamydial envelope, it has a fibrillar nucleoid, is non-infectious and metabolically active.

One of the first biochemical events to occur after the uptake of EBs is the reduction of disulphide bond cross-linking of native MOMP to its monomeric form. Hatch *et al.* (1986) demonstrated the reduction of the cross-linked MOMP, within 1 hour of entry of *C. psittaci* EBs into L cells, by the appearance of monomeric MOMP in electrophoretically separated sarkosyl extracts. The reduction of MOMP is inhibited by chloramphenicol but not by cycloheximide indicating that chlamydial, not host, protein synthesis is required for MOMP reduction (Hatch *et al.*, 1986). This suggests that a chlamydial protein component may facilitate the reduction of the envelope proteins (Section 1.5.1). The signal that triggers initiation of intracellular growth and multiplication is unknown, however, it is clear that reduction of the disulphide bond cross-linked MOMP is a critical early event in the differentiation of EBs to RBs. Several investigators have stimulated enzymatic

activity, usually seen in the RB, by treating host-free EBs with reducing agents. High resolution ^{31}P NMR was used to demonstrate ATPase activity in host-free EBs of trachoma and LGV biovars of *C. trachomatis* that had been reduced with 2-mercaptoethanol (Peeling *et al.,* 1989). In addition, EBs of *C. trachomatis* LGV biovar exhibit a DNA-dependent RNA polymerase activity that is doubled by treatment with 2-mercaptoethanol (Crenshaw *et al.,* 1990). It would seem that the reduction of disulphide cross-linking is sufficient to mimic EB-RB transition. It has been postulated that the reduction of MOMP allows it to form channels for the passage of ATP and other metabolites from the host cell into the newly ingested chlamydial cells (Bavoil *et al.,* 1984), however, this will be discussed more fully in Section 1.8.6.

1.4.3 Multiplication

By 8-12 hours post-infection, almost all EBs have differentiated into RBs. Once mature RBs have appeared, multiplication occurs by binary fission As bacterial multiplication proceeds, the inclusion membrane must expand to accommodate up to 100 progeny. While it is assumed that the membrane surrounding newly internalised bacteria is derived from the host plasma membrane, the source of membrane forming the expanding inclusion is unclear.

1.4.4 Inclusion membrane

Despite EB entry of host cells through an endocytic mechanism, the inclusion fails to fuse with the terminal compartment of the endocytic pathway, the lysosome. This evasion of lysosomal fusion was demonstrated by the absence of acid phosphatase, a lysosomal marker enzyme, within live *C. psittaci*-laden

inclusions (Heinzen *et al.,* 1996). Purified EB envelopes have also been shown to avoid lysosomal fusion while heat-killed chlamydiae are directed to vacuoles containing acid phosphatase. This data supports a role for chlamydial surface components in directing intracellular trafficking.

Using fluorescent pH-sensitive probes conjugated to chlamydiae, Schramm *et al.* (1996) directly measured the pH of *C. trachomatis*-containing vesicles. The pH remained above 6 for up to 12 hours post infection, while vacuoles containing heat-killed organisms fell to pH 5.3. The absence of significant vacuolar acidification was partially attributed to chlamydial reliance on the activity of Na^+/K^+-ATPases rather than H^+-ATPases for intracellular survival and replication. However, the small drop in pH from 7 to 6 has been suggested to be sufficient to catalyse the conversion of EBs to replicative RBs, and trigger the recycling of receptors. Schramm hypothesises that the avoidance of acidification may be a determining factor in the failure of the inclusion to complete the endocytic pathway and fuse with the lysosome.

It is accepted that survival of chlamydiae within the host is dependent upon inhibition of lysosomal fusion, however, the mechanism of avoiding fusion has yet to be determined. It has been suggested that chlamydiae remove themselves entirely from the endocytic pathway thereby avoiding their destruction by lysosomes. Evidence for this hypothesis is based largely on the use of probes demonstrating the absence of early or late endosome components and fluid phase markers in or around the inclusion. If the chlamydial inclusion is, indeed, removed from the endocytic pathway the question of how it sequesters nutrients and other requirements for growth from the host cell remains. Hackstadt *et al.* (1996) propose that the inclusion interrupts an exocytic pathway, receiving host-derived lipids and possibly

other nutrients by fusing directly with golgi-derived vesicles. This is supported by the demonstration that the inclusion and individual bacteria incorporated sphingomyelin-NBD endogenously synthesised in the golgi apparatus from C6-NBD ceramide, a fluorescent analogue of ceramide (Heinzen *et al.,* 1996). In addition, antibody probes for golgi-specific proteins were found to closely associate with the chlamydial inclusion. In contrast, C6-NBD-sphingomyelin introduced directly into the host plasma membrane was not delivered in significant amounts to the chlamydial inclusion. This indicates that the primary route of sphingomyelin delivery to the inclusion is by anterograde vesicular traffic directly from the Golgi apparatus rather than from the plasma membrane as a result of fusion with endocytic vesicles. Correlating these results, Hackstadt and co-workers propose that the inclusion acts as an aberrant Golgi-derived vesicle and as such is not recognised by the host cell as a vesicle of the endocytic pathway destined to fuse with lysosomes.

In direct contradiction to this work, van Ooij *et al.* (1997) have recently reported that markers of the endocytic pathway do indeed interact with both early and late forms of the inclusion. Using identical antibodies, cell line and *C. trachomatis* serotypes utilised by Hackstadt and colleagues, they found an accumulation of both the transferrin receptor and the cation-independent mannose-6-phosphate receptor, markers of early and late endosomes respectively, around the inclusion. However, no evidence of fluid phase transfer could be found. Further evidence of an interaction between the inclusion and the endocytic pathway was the reduction in the rate of transferrin recycling in infected cells in comparison to uninfected cells. Again these results contrast with those of Scidmore *et al.* (1996) who found no reduction in the rate of transferrin recycling in *Chlamydia*-infected cells. In light of these results van Ooij and co-workers believe that the chlamydial

inclusion remains part of the endocytic pathway interacting additionally with elements of the exocytic pathway. They propose that avoidance of lysosomal fusion is achieved by arresting the maturation of the inclusion along the endocytic pathway, a technique employed by several inclusion-dwelling intracellular parasites.

Expansion of the inclusion membrane is unaffected by cycloheximide-mediated inhibition of host protein synthesis (Ripa and Mardh, 1977) which suggests that protein components of the inclusion membrane do not result from *de novo* host protein synthesis. Tarasaka and co-workers (1996) reported that antibodies produced against isolated inclusion membranes from *Chlamydia* infected mouse cells solely recognise *Chlamydia*-derived proteins. Indeed, a 39 kDa chlamydial protein (Inc A) has been identified which is released from the RB and found to localise in the inclusion membrane (Rockey *et al.,* 1995). In addition, 3 more inclusion membrane localised proteins have been identified in *C. psittaci* termed Inc B-D (Scidmore *et al.*, 1998). The function of these proteins is, as yet, unknown.

The inclusion membrane constitutes a barrier between the replicating bacteria and the nutrient-rich environment of the host cytoplasm. It is clear that parasites existing exclusively within an intracellular vacuole must have a mechanism for host nutrient acquisition. As discussed previously, it is believed that the chlamydial inclusion may not be involved in the endosomal/lysosomal pathway and therefore is unlikely to fuse with nutrient-filled vesicles of that pathway. Heinzen and Hackstadt (1997) determined that the inclusion membrane of *C. trachomatis* was not passively permeable to low molecular weight compounds, a mechanism exploited by several protozoan parasites. Using fluorescent tracer molecules introduced into the cytoplasm of infected cells, they were unable to

determine any passive diffusion through the inclusion membrane. Alternatively, the inclusion may employ specific membrane transporter proteins to transfer nutrients from the cell cytoplasm to the lumen of the vacuole. This mechanism of nutrient acquisition would be unique amongst inclusion dwelling intracellular parasites, however, it is tempting to suggest that proteins within the inclusion membrane, such as IncA-D, may facilitate specific nutrient acquisition.

1.4.5 Energy metabolism

Moulder (1962) was the first to hypothesise that the *Chlamydia* are energy parasites, incapable of generating their own energy source. Evidence supporting this theory was largely circumstantial. Several researchers noted the absence from chlamydiae of key enzymes essential in the synthesis of ATP (Weiss 1965; Moulder *et al.,* 1965; Vender and Moulder, 1967). The requirement of an exogenous source of ATP and other nucleotides for macromolecular synthesis represent more positive evidence for the 'energy parasite' hypothesis (Moulder 1962; Hatch *et al.,* 1982; Hatch *et al.,* 1985). Weiss and Wilson (1968) found that an exogenous source of ATP either stimulated or was required by isolated chlamydiae for carbohydrate catabolism and the incorporation of low molecular weight substrates into lipids. In addition, Sarov and Becker (1971) noted that host-free synthesis of RNA required the addition of ATP. Hatch *et al.* (1982) directly demonstrated that host-free RBs of *C. psittaci* could transport ATP by a ADP-ATP exchange mechanism. ADP was a competitive inhibitor of ATP transport, however, other nucleotides had no effect. No transport of ATP was evident in EBs. Hatch and co-workers incubated RBs in phosphate buffer and noted that transported ATP was quickly hydrolysed to ADP by a magnesium-dependent ATPase. Hydrolysis of the ATP resulted in the energising

of the chlamydial membrane. These researchers hypothesise that *Chlamydia* hydrolyse ATP to generate a proton motive force which drives the acquisition of other host nutrients, such as lysine. Another putative method of ATP transport is discussed in Section 1.8.6. However, the recent sequencing of the entire *C. trachomatis* serovar D genome by Stephens *et al.* (1998) has revealed that *Chlamydia* possess the majority of the enzymes present in the glycolytic, pentose phosphate and tricarboxylic acid pathways. The identification of these enzymes indicate that, in addition to obtaining ATP from the host cell, *Chlamydia* are capable of producing their own energy source.

1.4.6 RB to EB transition

By 18 to 20 hours post-infection, most RBs within the inclusion have commenced re-organisation back into the infectious EBs. The RBs progress through several intermediate developmental forms before becoming mature EBs. Structural changes occurring during the re-organisation of RBs include a reduction in size, internal condensation, the formation of electron-dense nucleoids and the rigidification of the cell walls. RBs tend to cluster close to the inclusion membrane, where they are near to host cell mitochondria and endoplasmic reticulum.

In maturing from RB to EB, the chlamydial envelope undergoes dramatic changes. MOMP becomes extensively disulphide bond cross-linked forming high molecular weight cross-linked oligomers with the cysteine rich proteins (Newhall and Jones, 1983; Bavoil *et al.,* 1984) (Section 1.5.1). It is not clear if the formation of intracellular disulphide bonds is mediated by specific chlamydial enzymes or by a non-enzymatic mechanism.

The signal that triggers differentiation of the RB to the EB is unknown. cAMP and cGMP act as regulatory signals in many prokaryotic and eukaryotic cells and it has been proposed that these cyclic nucleotides are involved in the regulation of RB to EB transition. Significantly, RBs have a cAMP-binding protein which is absent from the mature EB. Kaul and Wenman (1986) observed that when HeLa cells are infected with the trachoma biovar, cAMP reversibly inhibits the conversion of RBs to EBs. When synthesis of MOMP is used as a marker for general protein synthesis, cAMP abolishes the increased rate of MOMP synthesis normally witnessed at 18-36 hours post-infection. The action of cAMP appears to be at the level of transcription. It has been observed to decrease the concentration of MOMP mRNA in *C. trachomatis* LGV-infected HeLa cells, and blocks the transcription of a recombinant MOMP gene *in vitro* (Kaul *et al.,* 1992). It would appear that cAMP acts as an inhibitor of RB-EB transition, and it has been hypothesised that this developmental process may be under bi-directional cyclic nucleotide control with cGMP acting as a stimulator.

1.4.7 Release from the host cell

As with modes of chlamydial entry, there seems to be several mechanisms by which chlamydiae can be released from the host cell. The method of release seems to vary according to species, strain and host cell-type. One mode of release shared by LGV and trachoma biovars of *C. trachomatis,* in addition to *C. psittaci* 6BC, is lysis of the host cell. In a time-lapse micro-cinematographic study of the trachoma biovar in McCoy cells, inclusions were seen to burst while apparently still inside the host cells (De La Maza and Peterson, 1982). Subsequently the host cells lysed and chlamydiae diffused away from the cell debris. Intact inclusions are also

released from host cells. De la Maza and Peterson (1982) used scanning electron microscopy to study the release of *C. trachomatis*-containing inclusions from McCoy cells. The host cell membranes were observed to distend, finally rupturing releasing both chlamydial inclusion and McCoy cell nuclei. In contrast, some host cells were seen to form a focal distension of their cytoplasmic membranes allowing the inclusion to be expelled from the cell without apparent harm to the rest of the cell surface.

The study of this particular area of the chlamydial lifecycle has been hampered as the mechanisms of release from specialised cells and tissues of intact host cells are undoubtedly more complex than in cell culture monolayers. To overcome this problem, Doughri *et al.* (1973) observed the release of a polyarthritis strain of *C. psittaci* from the intestinal epithelial cells of calves. At least three distinct modes of release were noted using this model: rupture of the infected cells, expulsion of whole infected cells into the intestinal lumen, and exocytosis of large chlamydiae-containing vesicles.

1.5 Architecture of the chlamydial envelope

1.5.1 Components of the chlamydial envelope

Experimental determination of the location and interaction of proteins within the chlamydial envelope structure are hampered by the biphasic developmental lifecycle of *Chlamydia*. Due to the asynchronous nature of events in the growth cycle, no preparation of EBs is likely to be totally free of RBs and intermediate forms of unknown stability. Therefore usual methods designed to detect surface exposure of proteins, such as radio-iodination, must be interpreted with caution.

Chlamydial envelopes were believed to be atypical in that they appeared to lack a traditional peptidoglycan layer. Using gas chromatographic mass spectrometry, Fox *et al.* (1990) could detect no muramic acid in purified EBs of *C. psittaci* or *C. trachomatis*. N-acetyl muramic acid forms links with N-acetyl glucosamine in the glycan backbone of peptidoglycan and, as it has been found in all gram-negative bacteria to date, is a good indication of the presence of peptidoglycan. Supporting observations include the failure to detect a peptidoglycan layer by electron microscopy and the failure of antibodies directed against peptidoglycan to react with *Chlamydia* (How *et al.*,1984; Hatch *et al.*, 1984). Paradoxically Barbour *et al.* (1982) demonstrated that *C. trachomatis* contains 3 penicillin-binding proteins, enzymes associated with the synthesis of peptidoglycan. This perplexing area has been further confused by the discovery of 20 genes bearing significant homology to enzymes involved in the peptidoglycan synthetic pathway (Stephens *et al.*,1998). It is now evident that *Chlamydia* have the potential to synthesise some form of peptidoglycan, perhaps at very low levels. The question remains - what would be the function of this small quantity of peptidoglycan, presumably not sufficient to form a complete sacculus? Despite the apparent deficiency of a complete peptidoglycan sacculus, EBs are found to be osmotically stable, however, RBs are readily lysed. It is proposed that chlamydial envelope proteins form a disulphide crosslinked supramolecular structure, a functional equivalent of a sacculus, providing osmotic stability to *Chlamydia* in extracellular environments (Newhall 1987).

The predominant proteins in the chlamydial envelope, and those believed to be involved in the disulphide crosslinked complex are the MOMP, one large cysteine rich protein (CRP) at 60 kDa termed Omp2, and a small CRP with a

molecular mass of 12 kDa and called Omp3. Hatch *et al.* (1981) were the first to report that MOMP of *C. psittaci* EBs was insoluble in SDS in the absence of a reducing agent, and suggested that disulphide bonds might account for the maintenance of the structural integrity of the organism. Newhall and Jones (1983), however, were the first to propose the disulphide cross-linkage of proteins as a substitute for peptidoglycan by demonstrating that MOMP forms inter-peptide crosslinks to itself in *C. trachomatis* EB preparations. Following this, it was reported that EBs of *C. psittaci* possess a 12 kDa and a 60 kDa cysteine rich protein, which runs as a doublet (Hatch *et al.,* 1984). These proteins are absent from the logarithmically dividing RBs. Hatch and co-workers (Hatch *et al.*, 1981) have demonstrated that the CRPs and MOMP are so extensively disulphide crosslinked in EBs that they fail to enter into a stacking gel during SDS-PAGE, under non-reducing conditions after boiling.

Newhall (1987) detected the synthesis of the MOMP and the 60 kDa CRP of *C. trachomatis* by 18 h postinfection. The 12 kDa CRP was not detectable until 30 h into the lifecycle. In contrast, while studying *C. psittaci*, Hatch *et al.* (1986) did not detect synthesis and incorporation of the CRPs until the RBs had begun to re-organise into EBs at 20-22 h post-infection. In this study, synthesis and incorporation of MOMP were detected early in the infection cycle (12 h post-infection).

The mechanism by which chlamydial envelope proteins become crosslinked has not been determined. Hatch *et al.* (1986) found that the CRPs and MOMP remain in a largely reduced state as long as *C. psittaci* remains intracellular, but, spontaneously crosslink when host cells are lysed and EBs are exposed to the extracellular environment. However, Newhall and Jones (1983) reported the

formation of crosslinks in *C. trachomatis* late in the intracellular section of the lifecycle suggesting an enzymatic mechanism for disulphide bond formation. Recently, membrane associated and periplasmic protein disulphide isomerases have been discovered in gram-negative bacteria, and enzymes similar to these may mediate the crosslinking process. Indeed, the large CRP itself may serve this function as a potential sulphydryl oxidoreductase active site has been located in the protein within *C. trachomatis, C. psittaci* and *C. pneumoniae* (Everett and Hatch, 1991).

The genes encoding the CRPs of many chlamydial strains have been cloned and sequenced. The deduced amino acid sequence of the small CRP contains 15 cysteine residues, while the 557 amino acid sequence of the large CRP contains 37 cysteines (Everett and Hatch, 1991). The position of these cysteine residues are highly conserved in all species of *Chlamydia.*

The location of the CRPs in the chlamydial envelope is still the cause of much debate. Hydropathy plots and conformational analysis of the small CRP amino acid sequence indicates that it is likely to be directly embedded in a membrane. However, the 12 kDa protein was specifically labelled in host cells incubated with [^{3}H] palmitic acid and therefore may be membrane associated through a covalently attached lipid portion. This evidence, combined with a predicted signal peptidase II cleavage site in the small CRP deduced amino acid sequence, suggest the 12 kDa protein is associated with the outer membrane through a lipid modification. The small CRP appears to resemble the Braun lipoprotein of *Escherichia coli* which links the peptidoglycan layer to the outer membrane (Everett and Hatch, 1991).

The location of the large CRP remains unclear. The 60 kDa protein is insoluble in Triton X-114 and sarkosyl, and is not labelled by 3'-(trifluoromethyl)-3-(m-[I^{125}]iodophenyl)diazirine ([I^{125}]TID), a lipophilic agent (Everett and Hatch, 1995). This evidence combined with the presence of a predicted signal peptidase I sequence in the large CRP open reading frame (Allen and Stephens, 1989) suggest that this protein, unlike Omp3, is not associated with a membrane and may be translocated through the inner chlamydial membrane to the periplasm. Alternatively, there is convincing evidence indicating the surface exposure of the Omp2 CRP in both *C. psittaci* and *C. trachomatis*. Ting *et al.* (1995) demonstrated that Omp2 from *C. psittaci* GPIC binds to HeLa cells and contains a terminal trypsin site that is accessible in native EBs indicating surface exposure. In addition, Birkelund *et al.* (1996) used immunoelectron microscopy to show that the N-terminal portion of the 60 kDa CRP in *C. trachomatis* L2 was surface exposed.

1.5.2 The POMP family

Several researchers have identified a group of proteins in the region of 90 kDa which have recently been shown to be surface exposed and therefore are potential mediators of chlamydial invasion. Longbottom *et al.* (1998b) identified a unique multigene family encoding these 90 kDa proteins in the ovine abortion sub-type of *C. psittaci* termed POMPs (putative outer membrane proteins). Analysis of the genome has uncovered a homologous multigene family in *C. trachomatis* serovar D containing 9 members, while analysis of the partially sequenced *C. pneumoniae* genome has identified 18 open reading frames (ORFs) encoding homologues to the *C. trachomatis* POMPs (Stephens *et al.*, 1998). The demonstration that POMPs are surface exposed on *C. psittaci* (Longbottom *et al.*,

1998a) and the fact that homologues have been identified in *C. trachomatis* and *C. pneumoniae* has focused interest in these proteins. As yet a function has not been assigned, however, with so many variants this protein may play a crucial role in chlamydial pathogenesis. It is feasible that the POMPs are involved in the process of chlamydial attachment and entry into the host cell, which would make these proteins important vaccine candidates.

1.5.3 Chlamydial surface projections

Chlamydiae possess a unique ultrastructural feature, the presence of macromolecular surface projections that extend from the cytoplasmic membrane and range from 45-90 nm in length. These projections average 6 nm in diameter with a 10 nm bulge at one end. On the EB surface, the projections are hexagonally arranged with each EB displaying approximately 18-20 projections (Matsumoto, 1982). At the base of each projection is a nine component 'rosette' of unknown origin.

These surface projections are also present on chlamydial RBs and have been shown by electron microscopy to associate with the inclusion membrane thus prompting suggestions of a possible role in nutrient acquisition. Most recently, Bavoil and Hsia (1998) have hypothesised that the surface projections and the rosette-like structures surrounding their base form Type III secretion apparatus and genes encoding Type III secretion machinery have been identified in *Chlamydia* (Hsia *et al.,* 1997; Stephens *et al.,* 1998). Similar type III secretion-associated structures have been observed in other bacteria (Galan *et al.*, 1992; Hueck, 1998).

1.5.4 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major surface antigen of *Chlamydia* (Nurminen *et al.*, 1983) and chemically resembles the Re type LPS of *Salmonella* species (Kochetkou and Knirel, 1993). Like that of other bacteria, chlamydial LPS is composed of a lipid A portion and a saccharide moiety which contains a genus-specific epitope composed of a unique 2-8 linkage of the linear 3-deoxy-D-manno-octulosonic acid (Kdo). The structure of this saccharide was established using the LPS from a recombinant *E. coli* transformed with the chlamydial *gseA* gene (Belunis *et al.*, 1992). The product of this gene is a multifunctional glycosyltransferase catalysing all three glycosylation steps in the assembly of the Kdo trisaccharide. Surprisingly, homology of *gseA* genes, at the nucleic acid and amino acid level, is low, despite this, the *Chlamydia*-specific determinant is conserved (Mamat *et al.*, 1993).

Chlamydial LPS is phenotypically of the rough type, however, recent data suggests that chlamydiae also produce a smooth LPS variant. Lukaccova *et al.* (1994) observed that smooth LPS was preferentially expressed in chlamydiae grown in the yolk sacs of embryonated eggs. In addition, smooth LPS was also detected by immunoflourescence in tissue culture-grown chlamydiae. These authors suggest that the ability of *Chlamydia* to phase shift between rough and smooth LPS synthesis, as observed in many other bacteria, would be highly advantageous in surviving the host cell immune system.

The significance of chlamydial LPS in infection is unclear. Several researchers have suggested that LPS may act in the protection of *Chlamydia* from the host immune system. Using immunoelectron microscopy, Birkelund *et al.* (1989) demonstrated that binding of LPS-specific monoclonal antibodies to the

chlamydial membrane stripped LPS from the surface. It is not clear if the unique 2-8 Kdo linkage of chlamydial LPS is intrinsically unstable, resulting in the breakage of the linkage upon antibody binding. However, as LPS is immunogenic, it has been hypothesised that chlamydial LPS may be sacrificed to secure the survival of the chlamydiae. In addition, chlamydial LPS has been found to accumulate in the plasma membranes of infected host cells. Karimi *et al.* (1989) proposed that the incorporation of chlamydial LPS in eukaryotic plasma membranes reduces membrane fluidity and makes infected host cells less susceptible to cytotoxic T-cell attack.

Despite the high levels of anti-LPS antibodies resulting from chlamydial infection, these antibodies are not found to be protective. A possible explanation for this anomaly was proposed by Salinas *et al.* (1994). Using immunogold labelling they demonstrated that LPS of *C. psittaci* EBs localised to the inner side of the external membrane, while LPS localised to the outer side of the RB membrane. The internal localisation of LPS in EBs, shown in this study, could explain the lack of neutralising LPS-specific antibodies.

1.5.5 Other potential envelope proteins

Our understanding of the protein components that make up the chlamydial outer membrane is largely based on experimental designs that employ detergent extraction of non-reduced EBs. This approach eliminates non-cysteine crosslinked outer membrane proteins from analysis. In addition, the use of monoclonal antibodies to probe for surface-exposed proteins positively selects for the dominant proteins of the outer membrane. Due to the prohibitively small amounts of antigen available from tissue culture growth of *Chlamydia*, researchers have been restricted

to these analytical techniques. The results of such experiments may lead us to believe that the envelope of *Chlamydia* is a homogenous mass of MOMP, however, this is not the case. Using techniques such as (1) radio-iodination of the EB surface (2) sensitivity upon exposure to protease or (3) presence in COMC preparations, proteins of 155, 96, 45 and 30 kDa have been suggested to associate with the chlamydial envelope.

Batteiger *et al.* (1993) used COMC from the GPIC strain of *C.psittaci* to analyse their effectiveness in eliciting immunity in a guinea-pig genital model. In addition to the ubiquitous MOMP and 60 kDa CRP, the resultant anti-sera also reacted with putative envelope proteins of 84, 72, 47 and 35 kDa. These OG-DTT-solubilised COMCs provided protection from genital challenge, however, it is unclear if this is purely as a result of the protective role of MOMP.

In *C. trachomatis*, a 27 kDa protein with homology to the *Legionella pneumophila* macrophage infectivity potentiator (MIP) protein has been characterised (Lundemose *et al.,* 1992). This protein has a peptidyl-prolyl cis/trans isomerase activity, which, when inhibited, interferes with the early stages of chlamydial development. The chlamydial MIP-like protein is poorly surface-exposed and specific antibodies are non-neutralising in the absence of complement. However, the amino acid sequence of the MIP-like protein contains a signal peptidase II recognition sequence and, like the 12 kDa CRP, is thought to be a lipoprotein associating with the inner chlamydial envelope.

Several chamydial proteins have been identified that share some homology to members of the heat shock protein (hsp) family (Cerrone *et al.,* 1991; Ho and Zhang, 1994). Surprisingly, an envelope association has been reported for a hsp70 homologue, a DnaK-like homologue. Raulston *et al.* (1998) demonstrated that a

recombinant of the hsp70 homologue of *C. trachomatis* attaches to epithelial cells and associates with the chlamydial outer membrane. Zhong and Brunham (1992) have also shown that certain *C. trachomatis* hsp70 antibodies neutralise infectivity *in vitro*. Whether or not this hsp-like protein acts as an adhesin or a chaperonin in the adherance process is unclear.

1.6 Genetics

The overall pace of research into this bacteria is slowed by the absence of compatible genetic systems. Until now, there has been no well established, convenient system for the removal, manipulation and re-introduction of genetic material in chlamydiae. In addition, chlamydial genes do not express well in other hosts. However, O'Connell and Maurelli (1998) have recently reported the introduction of foreign DNA into the Cal 10 strain of *C. psittaci*. Engineering shuttle plasmids containing control elements of the previously sequenced plasmid of *C. trachomatis* L2 and which allow expression in *E. coli*, these researchers were able to achieve the stable expression of chloramphenicol resistance in Cal 10. Interestingly, these successful transformants did not result from transfection with the specially designed shuttle plasmids but from an *E. coli* vector containing the resistance gene. Although this work is in its infancy it marks a significant advancement in *Chlamydia* research and offers hope that a convenient genetic system is achievable in the foreseeable future. The introduction of mutations by this method would accelerate our understanding of these complex bacteria. Until that time, recombinant DNA techniques are the primary methods by which to study the chlamydial structural genes.

1.6.1 Genome

Chlamydia have one of the smallest prokaryotic genomes consisting of a 1045 Kbp circular chromosome, approximately ¼ of the size of the *E. coli* chromosome. Assuming non-overlapping ORFs and no bi-directional transcription, the chlamydial chromosomes have the capacity to encode from 400 to 600 proteins. The sequencing of the *C. trachmatis* serovar D genome has recently been completed as has the sequencing of the *C. pneumoniae* genome (Stephens *et al.,* 1998). Already this data has had a major impact on the study of *Chlamydia*, challenging many of our previous beliefs. Access to the genome sequences of these two chlamydial strains will initiate new research, dramatically increasing our understanding of these complex bacteria. It is hoped that sequencing of the *C. psittaci* genome will be achieved in the not too distant future.

Lovett *et al.* (1980) were the first to observe and isolate plasmids from *Chlamydia*. Endonuclease restriction and Southern analysis indicated a significant lack of DNA homology between *C. trachomatis* and *C. psittaci* plasmids. However, endonuclease studies of several *C. trachomatis* serovars demonstrated that these plasmids were highly conserved. High sequence homology implies an essential role for these plasmids in the virulence or biology of *Chlamydia*. Indeed, plasmid specific RNA has been found to be transcribed throughout the developmental cycle. However, the plasmid is not essential for chlamydial growth or disease (Peterson *et al.,* 1990). Chlamydial plasmids have subsequently been sequenced (Sriprakash and MacAvoy, 1987; Hatt *et al.,* 1988; Black *et al.,* 1989) and the expression of several genes has been investigated (Pearce *et al.,* 1991; Comanducci *et al.,* 1993), unfortunately the function of the encoded products remains unclear.

The processes of differentiation between the EB and RB developmental forms are undoubtedly mediated at the genetic level. Two methods of genetic regulation are involved. First, regulation of the transcription of specific early and late genes is essential for the systematic differentiation of these morphologically distinct forms. The second process of genetic regulation is at the level of chromosome structure. These regulatory mechanisms will be discussed in turn.

1.6.2 Chromosomal regulation

During transformation of RBs to EBs the chlamydial chromosome has been observed to condense, resulting in the appearance of an electron dense body at the centre of the cell. In contrast, RB chromatin appears more pleomorphic resembling that seen in other bacteria. Recently, two lysine-rich proteins of 18 (Hackstadt *et al.,* 1991; Perara *et al.,* 1992) and 23-32 kDa (Hackstadt *et al.,* 1993) have been identified in *C. trachomatis* and found to share sequence homology with the eukaryotic histone H1-like proteins. They are thought to play a key role in chromosome compaction during RB to EB transition. Both proteins are expressed late in the lifecycle at a time when chlamydial DNA is undergoing condensation accompanied by down-regulation of transcription and metabolic processes (Hackstadt *et al.,* 1991; Perara *et al.,* 1992).

The best characterised of these is the 18 kDa protein designated Hc1, which is conserved among all *C. trachomatis* serovars as well as *C. psittaci* (Peterson *et al.,* 1988). The chlamydial gene encoding Hc1, *hctA*, has been cloned and sequenced. Expression of this gene in *E.coli*, revealed that the nucleoid of the Hc1-expressing bacteria was highly condensed in comparison to the non-expressing control, ultrastructurally reminiscent of corresponding structures in intermediate

developmental forms of *C. trachomatis* (Barry *et al.,* 1992; Barry *et al.,* 1993). These results suggest Hc1 is sufficient to induce compaction of chromatin and may mediate the higher order nuclear structure of EBs.

It is clear Hc1 initiates these chromatin structural changes by directly binding to DNA. Purified recombinant Hc1 has been demonstrated to co-operatively bind double stranded DNA *in vitro*, forming a condensed spherical Hc1-DNA complex (Christiansen *et al.,* 1993). The formation of these complexes seems largely independent of DNA concentration but highly dependent on protein concentration, indicating co-operative binding of Hc1 to DNA. This DNA binding facility was found to be restricted to the carboxyl region of Hc1 (Remacha *et al.,* 1996). However, compaction of the nucleoid is not complete in the absence of the N-terminal domain.

In addition to the structural role in the condensation of the nucleoid, expression of Hc1 in *E. coli* is self limiting and down regulates transcription, translation and replication at concentrations similar to those observed in chlamydial EBs (Barry *et al.,* 1993). However, expression of Hc1 at sub-structural levels results in a relaxation of the superhelical DNA structure accompanied by marked changes in gene expression. These results suggest that Hc1 may have many discrete functions including the inhibition of replication, transcription and translation by initiating the condensation of the nucleoid structure. This may be preceded by a more subtle effect on gene expression resulting from a general relaxation of the superhelical structure of the chromosome.

1.6.3 Transcriptional regulation

The mechanisms that trigger and regulate gene expression in chlamydiae have not been identified but are likely to occur at the level of transcription initiation. Sarov and Becker (1971) were the first to reveal that EBs synthesise RNA using a DNA-dependent RNA polymerase by observing the effects of the general RNA polymerase inhibitor rifampin on differentiating EBs. RNA synthesis, courtesy of this enzyme, is an early event in the chlamydial lifecycle, RNA being detected at 3 hours post-infection (Pollard *et al.,* 1960). The polymerase molecule is a multi-subunit enzyme composed of α, β, β' and σ subunits (Koehler *et al.,* 1990; Engel *et al.,* 1990). The core enzyme, $\alpha_2\beta\beta'$, is a non-specific DNA binding protein. Association of the σ subunit with the core enzyme enables sequence specific DNA recognition permitting the specific binding of the enzyme to the promoter sequences.

Initial attempts to understand the mechanisms of regulation of gene expression addressed the role of the σ subunit. Using monoclonal antibodies and PCR techniques, Engel and Ganem (1990) were able to identify and clone only one chlamydial σ subunit, identified as σ^{66}. However, the *Chlamydia* Genome Project (Stephens *et al.,* 1998) has recently identified two additional ORFs with homology to known sigma factors. Interestingly, high sequence homology exist between the σ^{66} subunit and the σ^{70} subunit of *E. coli* (Birkelund *et al.,* 1988). Detailed comparison reveals striking homologies in domains 2 and 4 (responsible for -10 and -35 promoter region recognition and binding) with 93% and 67% identity respectively (Mathews and Sriprakash, 1990). This level of homology is perplexing when we consider that chlamydial genes typically do not have *E. coli*-like consensus

promoter elements. Indeed, chlamydial promoter sequences appear to be different from those of other prokaryotes, so much so that few chlamydial promoters tested so far can function efficiently in *E. coli*. *In vitro* assay of promoter recognition requirements using the σ^{66} subunit reveal that chlamydial RNA polymerase can tolerate considerably more variation at the -10 and -35 regions (Mathews and Sriprakash, 1994; Douglas and Hatch, 1996). This suggests that promoter region context and, perhaps, structure may play a significant role in the transcription.

Due to the apparent lack of consensus promoter sequences in the regions 5′ to the structural genes, most chlamydial genes are not efficiently expressed in *E. coli*. Without the identification of a defined and recognisable promoter our understanding of transcriptional regulation in *Chlamydia* is limited. Presently, promoters described for *Chlamydia* are based solely on the assumption that they are immediately adjacent to the 5′ mRNA region as determined by S1 mapping and primer extension. However, these techniques are prone to error.

1.7 Chlamydial vaccine development

Vaccines against the *C. psittaci* OEA strain represent the most successful application of immunoprophylaxis for any disease of chlamydial aetiology. A series of studies testing various forms of experimental vaccines culminated in the development of an inactivated whole organism vaccine which formed the basis of a commercial product (McEwen *et al.*, 1951a; McEwen *et al.*, 1951b; Foggie 1952). This vaccine consisted of a crude suspension of egg-cultured chlamydial organisms suitably inactivated and formulated as an emulsion in oil. Although not entirely eliminating the disease, the vaccine reduced the incidence of OEA to an

economically acceptable level for many years. In recent years, OEA has re-emerged to become a prevalent cause of infectious lamb loss due to a reduction in the efficacy of the commercially available vaccine (Linklater and Dyson, 1979).

The antigenic properties of MOMP have been studied in detail since the landmark discovery that MOMP purified from SDS gels was capable of raising antibodies which could neutralise the infectivity of *C. trachomatis in vitro* (Caldwell and Perry, 1982). Vaccine preparations based on chlamydial outer membrane complexes, which are highly enriched for the MOMP in its native form, have been shown to be protective against chlamydial disease in sheep (Tan *et al.,* 1990), guinea pigs (Batteiger *et al.,* 1993), and mice (de Sa *et al.,* 1995; Pal *et al.,* 1997). However, experimental vaccines based on denatured or non-native recombinant MOMP preparations have yielded, at best, only partial protection (Pal *et al.,* 1997).

These results clearly make MOMP the primary candidate for a sub-unit vaccine against chlamydial infection and current research into the design of such a vaccine has focused on the use of highly immunogenic oligopeptides and cloned recombinant MOMP fragments (Su and Caldwell, 1992; Murdin *et al.,* 1993; Zhong *et al.,* 1994). The observation that neutralising mAbs specific to MOMP recognise either linear or conformational epitopes, prompted the use of oligopeptides to mimic neutralising B-cell epitopes of MOMP. Ideally, the chosen B-cell epitope should be widely recognised and contain an appropriate T-cell epitope which will serve to elicit an immunological memory. In addition, candidate peptides need to be delivered in such a way as to induce a potent mucosal immune response, by the production of IgA and IgG antibodies, and a swift recall response after the organism is encountered. Results using oligopeptide based vaccines have been disappointing,

perhaps because the immunogens do not induce protective cellular and humoral immune responses recalled by native epitopes on the organism. This suggests that a successful vaccine may need to incorporate both sequential and conformational information in order to elicit a protective immune response.

The future of chlamydial vaccine development may lie with the relatively new technique of DNA vaccination (Lui, 1995). DNA vaccination provides protective immunity following the expression of the foreign protein by the hosts expression machinery. The utilisation of the host expression systems allows the foreign protein to be presented to the host immune system in a more recognisable way, analogous to that which occurs during natural infection by an intracellular pathogen. Using a murine model of pneumoniae, induced by the mouse pneumonitus isolate of *C. trachomatis*, Zhang *et al.* (1997) reported a protective immune response after intra-muscular DNA immunisation with the chlamydial gene encoding the MOMP. The MOMP DNA vaccine resulted in the production of significant levels of protective immunity from lung challenge, however, sterile immunity was not achieved. The authors have suggested that levels of protective immunity may be increased by the delivery of the vaccine to the mucosal surfaces mimicking the natural routes of infection. The results of this research are encouraging, however, the use of a anti-chlamydial DNA vaccine in humans may still be some way off. Indeed, Pal *et al.* (1998) recently reported that inoculation of Balb/c mice with a variety of eukaryotic expression vectors containing mature MOMP constructs or DNA from the four variable segments of MOMP from the mouse pneumonitis strain of *C. trachomatis* failed to protect against a genital challenge.

Clearly, significant problems stand in the way of an effective anti-chlamydial vaccine. In general, protective MOMP epitopes appear to be serovar specific therefore making the development of a universal vaccine for *Chlamydia* very difficult. In addition, the possible role of DTH in the pathogenesis of acute chlamydial infection make the choice of vaccine target crucial (Section 1.3). Despite these significant obstacles, the search for an effective vaccine continues to be a realistically achievable goal.

1.8 The major outer membrane protein

1.8.1 Structure

The MOMP is a surface exposed, integral membrane protein of approximately 40 kDa comprising, at least, 60% of the total outer membrane protein content (Caldwell *et al.,* 1981). Surface exposure of MOMP is indicated by the susceptibility of MOMP from EBs to trypsin digestion, radio-iodination and recognition by monoclonal antibodies. Baehr *et al.* (1988) have suggested that the MOMP is also exposed to the periplasm.

MOMP cannot be called cysteine-rich in comparison with other chlamydial envelope proteins, however, amino acid analysis has indicated that seven cysteine residues within the MOMP sequence are conserved in all 4 currently recognised species of *Chlamydia.* These conserved cysteines are thought play a crucial role in maintaining the structural integrity of the elementary body forming intra- and inter-molecular disulphide bonds (Section 1.5.1) (Newhall and Jones, 1983). Despite a relatively large proportion of MOMPs amino acids being designated as hydrophobic (43 % in *C. trachomatis* serovar L2) - charged, polar and hydrophobic residues are

distributed evenly throughout the sequence (Stephens *et al.,* 1986). This results in the lack of a linear hydrophobic sequence sufficiently long to form a transmembrane α-helix and is suggestive of a β sheet secondary structure.

Several bodies of work have suggested that MOMP may be post-translationally glycosylated. Swanson and Kuo (1991) reported that an N-linked carbohydrate was associated with MOMP. Isolated MOMP bound specifically to concanavlin A, wheat germ agglutanin and *Dolichos biflorus* agglutanin in a lectin binding assay. These lectins recognise α-D-mannose, D-N- acetylglucosamine and α-N-acetylgalactosamine. Determination of the glycan structure of MOMP was hampered by the difficulty in obtaining sufficient amounts of purified carbohydrate. However, using a sensitive 2-D sugar mapping technique, Kuo *et al.* (1996) analysed the structure of the MOMP glycan and found it to contain high mannose type oligosaccharides, and bi- and tri-antennary complex oligosaccharides with terminal galactose. Although convincing, these results do not determine the nature of the association between MOMP and its alleged glycan. It is possible that the oligosaccharide is not covalently bound to MOMP, merely associated by a non-covalent specific binding interaction. As discussed previously, MOMP is thought to specifically bind glycosaminoglycans, for example heparin, and such an interaction could account for the results of these researchers. Unpublished observations of this laboratory support this hypothesis (Herring, A.J., personal communication). Full length recombinant MOMP proteins are found to give an identical molecular mass on SDS-PAGE gels to that observed with the native protein. As it is unlikely that the recombinant MOMP is being post-translationally glycosylated by *E. coli,* glycosylation of native MOMP would result in an apparent difference in molecular mass.

There is a growing body of evidence suggesting that MOMP exists as an oligomer within chlamydial outer membranes. Several groups have shown MOMP forms disulphide bond cross-linked multimers, based on the original observation that MOMP could not be solubilised without the use of a reducing agent (Hatch *et al.,* 1981). Purified MOMP, solubilised in octyl-glucoside and reduced with dithiothreitol gives an apparent molecular mass, by SDS-PAGE analysis, almost 3 times that observed for the heat-denatured SDS-treated protein (McCafferty *et al.,* 1995; de Sa *et al.,* 1995). The observed molecular mass of 110 kDa is suggesting that MOMP forms trimers. The molecular mass estimated for non-denatured protein by SDS-PAGE analysis is only an apparent molecular mass and is very dependent on protein conformation. However, analysis of MOMP multimers by sucrose density gradient centrifugation indicated that the oligomers had an approximate sedimentation coefficient of 7.2 S - consistent with the value of 6.8 S for *E. coli* porin trimers (McCafferty *et al.,* 1995).

The maintenance of the oligomeric structure of MOMP may well be due to its close association with LPS, the only other component of chlamydial outer membranes proven to be surface exposed. Using the crosslinking agent disuccinimidyl selenodipropionate (SSP), Birkelund *et al.* (1988) were able to crosslink MOMP to LPS. MOMP oligomers were observed at 100 and 110 kDa, however, only in the presence of LPS. No polymeric complexes of MOMP without LPS were detected. It may well be the case that LPS is required to stabilise the multimeric structure of MOMP.

1.8.2 Sequence analysis

The gene for MOMP, termed *ompl* or *ompA*, was first sequenced in *C. trachomatis* serovar L2 and consisted of a 1182 bp open reading frame encoding a 394 amino acid protein containing 9 cysteine residues (Stephens *et al.,* 1986). The known amino terminal amino acid was preceded by a 22 amino acid signal sequence which conformed in structure to that of other outer membrane protein signal sequences.

Comparative analysis of the entire *ompl* sequence among several serovars of *C. trachomatis* demonstrated that the gene is 84-97% identical at the nucleotide and amino acid levels (Kaltenboeck *et al.,* 1993; Fitch *et al.,* 1993). Although nucleotide variation is distributed throughout the entire ORF, analysis of deduced amino acid sequences of MOMP genes from *C. trachomatis* and *C. psittaci* reveal that amino acid variation is clustered into 4 variable segments (VS I-IV) (Stephens *et al.,* 1987). These 4 variable regions intersperse five constant regions thought to be buried within the cell membrane. In contrast, the VSs are thought to be surface exposed due to their accessibility to antibodies and susceptibility to trypsin digestion and radio-iodination (Su *et al.,* 1988).

1.8.3 Antigenicity

The antigenic diversity of *C. trachomatis* and *C. psittaci* MOMPs has been attributed to the four sequence-variable segments (Stephens *et al.,* 1987; Stephens *et al.,* 1988b). Using murine monoclonal antibodies and overlapping synthetic peptides some epitopes have been mapped to specific regions of the MOMP (Stephens *et al.,* 1988b). With one exception (Conlan *et al.,* 1988), epitopes of all specificities have been mapped to one of the 4 variable regions. Available mapping

data largely focuses on the serovars of *C. trachomatis*. On the basis of these studies, it can be concluded that cross-reactive epitopes, such as species, subgroup and serogroup-specific epitopes reside in VS4 while serovar-specific epitopes reside in VS1 or VS2. VS IV is the largest of the hypervariable regions of *C. trachomatis* and *C. psittaci* domains and is located near the carboxy terminus of the protein. Different serovars of *C. trachomatis* have been distinguished both by direct sequence analysis of VS IV (Yuan *et al.,* 1990) and by restriction fragment length polymorphism (RFLP) analysis (Gaydos *et al.,* 1992a; Yang *et al.,* 1993). The identification of minor differences in the nucleotide sequence of this variable region have been employed to differentiate strains of the same *C. trachomatis* serovar from different individuals.

To date, only a few studies have examined the MOMP-encoding genes of the *C. pneumoniae* strains. Sequence comparison of entire *ompl* genes from two different isolates (AR 39 and IOL207) in regions comparable with VS IV of *C. trachomatis*, showed a high level of conservation (Gaydos *et al.,* 1992b). In contrast, to *C. trachomatis* and *C. psittaci*, the *ompl* sequences of *C. pneumoniae* isolates appear to be conserved. The reason for this lack of polymorphism is unclear, however it has been suggested that it is an indication of the evolutionary youth of *C. pneumoniae*. In addition, it has been proposed that the MOMP of *C. pneumoniae* is less immunogenic and antigenically complex. Serological results appear to be conflicting. Campbell *et al.* (1990) and Black *et al.* (1991) found weak or no reactivity of *C. pneumoniae* patient sera with MOMP by immunoblotting studies. At the 9th International Symposium on Human Chlamydial Infection, Christiansen *et al.* (1998) reported that they could not detect MOMP on the surface of *C. pneumoniae* EBs. These researchers hypothesised that proteins ranging from

97-99 kDa (homologous to the pomp family identified in *C. psittaci*, Section 1.5.2) may form a folded layer at the surface of the EB thereby protecting MOMP and other proteins from surface exposure. Paradoxically Persson and Peykani (1998) reported at the same meeting that the oligomeric form of MOMP from *C. pneumoniae* was recognised by many monoclonal antibodies specific to *C. pneumoniae* and that this polymeric form of MOMP was also reactive with human sera.

1.8.4 Neutralisation

Significantly, both monospecific polyclonal and monoclonal antibodies to epitopes located in VS1, 2 and 4 can neutralise chlamydial infectivity *in vitro* (Lucero and Kuo, 1985) and *in vivo* (Peterson *et al.,* 1988; Zhang *et al.,* 1987). In general it is believed that antibodies directed to serovar and subspecies-specific epitopes are protective, while those recognising species-specific epitopes are not, although there can be exceptions (Peeling *et al.,* 1984). Studies have shown that neutralisation is dependent on the binding of bivalent immunoglobulin G (IgG) (Su and Caldwell, 1991). Depending on host cell culture conditions, the mechanism of action appears to be due to the prevention of attachment of elementary bodies to the host cell or, in some cases, inhibition of a post-internalisation process. As bivalent IgGs were used in these neutralisation assays, antibody mediated aggregation of the chlamydial inoculum could not be excluded as a possible neutralisation mechanism. However, Su and Caldwell (1991) demonstrated that monovalent Fab antibodies to serovar and subspecies epitopes on VS1 could neutralise infectivity of *C. trachomatis* in HaK cells thereby removing aggregation as a possible mechanism of neutralisation. Confusion still surrounds the requirement of complement to achieve

neutralisation. Su *et al.* (1991) found that monovalent Fab fragments of a MOMP-specific mAb neutralised infectivity of an A-serovar of *C. trachomatis* in both HeLa and HaK cells, but intact IgG_3 caused neutralisation only in HaK. HeLa 229 cells were found to possess Fc receptors and the authors suggested that this may account for the inconsistencies observed in the requirement for complement to achieve neutralisation.

The demonstration that some neutralising mAbs recognise linear epitopes has concentrated efforts on designing a MOMP sub-unit vaccine based upon using oligopeptides or cloned recombinant fragments. However, it is believed that a successful peptide vaccine may require structural information (conformational epitopes) to elicit antibodies which maximally cross-react with native neutralisation epitopes on the organism. These results underline the importance of MOMP as the immunodominant antigen of *Chlamydia.*

1.8.5 MOMP genetic regulation

Using techniques such as primer extension and S1 nuclease analysis, Stephens *et al.* (1988a) proposed that the *ompl* gene of *C. trachomatis* L2 had two tandem promoters arranged upstream from the single copy structural gene. One of these promoters (P1) produced an mRNA transcript detectable at as little as 4 h post infection. The second promoter (P2) was responsible for production of a slightly larger transcript, appearing at 12 h post infection coinciding with the onset of binary fission. Stephens *et al.* (1988a) hypothesised that the smaller transcript was produced constituitively while the longer transcript was developmentally regulated perhaps to provide supplemental transcription as binary fission commenced. Interestingly, again using primer extension and S1 nuclease analysis, Yuan *et al.*

(1990) discovered that the GPIC and mouse pneumonitis strains of *C. psittaci* had 3 and 4 different sizes of *ompl* transcripts respectively. These varying transcripts were again attributed to transcription from multiple tandem promoters.

The identification of multiple tandem promoters is disputed by the work of Douglas and Hatch (1995). Again using primer extension these researchers identified two transcription initiation sites identical to those seen by Stephens *et al.* (1988a) in the L2 serovar of *C. trachomatis*. However, employing an alternative technique where the two putative promoter sites are cloned into a transcription assay vector only promoter P2 initiated transcription. Tandem transcript bands were identified from this cloned promoter, however, they are believed to result from imprecise termination of the transcript. Douglas and Hatch (1995) propose that P2 is the only active *ompA* promoter generating the longer transcript.

1.8.6 Function

With such a predominance in the chlamydial outer membrane, the function of MOMP has been the focus of much attention. However, problems purifying native MOMP or obtaining recombinant MOMP in a native form have hampered the study of structure/function relationships for this protein. Despite this, MOMP is thought to be multifunctional with a role both in the infectious process (Section 1.4.1) and in the maintenance of structural rigidity via disulphide bond cross-linking within the EB outer membrane (Hatch 1996; Newhall 1987) (Section 1.5.1).

Perhaps the most convincing proposed function of MOMP is that of a chlamydial porin. MOMP shares many biochemical properties with gram negative porin proteins. The general amino acid composition of MOMP is very similar to the porins of *Neisseria gonorrhoea* and *E. coli*. In addition, MOMP forms SDS-

resistant oligomers (Section 1.8.1), has an acidic pI and has a molecular mass within the range of 30-40 kDa, all properties common to porins. To date, the only research to directly test this porin channel hypothesis was that of Bavoil *et al.* (1984). Using the technique of liposome swelling, they demonstrated that chlamydial outer membrane complexes of *C. trachomatis* contained water-filled pores. Due to the overwhelming presence of MOMP, in comparison with other proteins, in such outer membrane preparations it was assumed that MOMP was the likely pore-forming protein.

It is clear that MOMP's pore-forming activity would be primarily utilised at the intracellular, RB stage of the chlamydial lifecycle. Bavoil *et al.* (1984) proposed that the reduction of the outer membrane disulphide bonds, during EB to RB transition, 'opened' chlamydial pores allowing the uptake of ATP and other nutrients. Evidence supporting this hypothesis included activation of the COMC pores by treatment with dithiothreitol, and the blocking of re-oxidation with iodoacetimide.

1.9 Aims and objectives

The wide ranging objective of this study was the structural and functional characterisation of the MOMP of the OEA isolate of *C. psittaci*. More specific aims were to fully investigate the MOMP porin hypothesis of Bavoil *et al.* (1984) at the molecular level, using planar lipid bilayer reconstitution and, at the structural level, by CD analysis of the purified protein. In order to achieve these specific objectives it was necessary to develop methods of purification which would yield useful amounts of structurally native MOMP. It is hoped that the assignment of a definite

function to MOMP, in addition to more detailed structural information, will help and improve the design of future MOMP-based anti-chlamydial vaccines.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chlamydial culture and growth

The ovine abortion isolate of *C.psittaci,* S26/3, was used throughout this study (McClenaghan *et al.,* 1984). Mycoplasma-free McCoy cells were used for the cell culture of chlamydial organisms. Cells were grown in 225 cm^2 flasks in RPMI 1640 (Gibco) supplemented with 5% (v/v) newborn calf serum (NBC), 0.2% (w/v) sodium bicarbonate, 1% (w/v) HEPES, 0.1 mg/ml Streptomycin, 25U/ml Nystatin and 5µg/ml Gentimicin. Confluent monolayers were obtained after approximately 3 days. Cells were infected with *Chlamydia* by incubation with RPMI, supplemented as above, containing 1% (v/v) chlamydial inoculum (5 x 10^7 inclusion forming units (IFU)), transport medium (0.2 M sucrose, 7 mM K_2HPO_4, 4 mM KH_2PO_4, 5 mM L-glutamic acid, 0.0016% (w/v) phenol red, 10% (v/v) foetal bovine serum (FBS), 0.05 mg/ml Gentimicin, 0.2 mg/ml Streptomycin and 25 U/ml Nystatin) and 1µg/ml of cycloheximide. Flasks were incubated at 37°C for 3-7 days. This inoculum was produced from an isolate of *C. psittaci* S26/3 recovered from a field outbreak of ovine abortion and grown in yolk sacs of day old chick embryos, the isolate was further passaged in tissue culture, harvested and stored, as described above, in liquid nitrogen. Chlamydial cultures used for the purification of MOMP, were harvested and stored at -70°C. Cultures used as inocula were harvested at approximately 3 days post-infection and stored in sterile transport medium at -70°C.

2.2 SDS-PAGE

Discontinuous SDS PAGE was carried out as described by Laemmli (1970) using 12.5% or 10% mini slab gels (Mini Protean II, BioRad) run at a constant voltage of 200 V for approximately 1 h. 12.5% large format gels (Protean II,

BioRad), used to prepare samples for electroelution, were run overnight at 100 V constant voltage. Samples were dissolved in Laemmli sample buffer containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol (Sigma), and boiled for 3 min or left unboiled prior to loading. A range of molecular mass standards were included in each run, the 'Electran' calibration kit for SDS-PAGE (BDH) with molecular weights ranging from 12.5-78 kDa, the Mark 12 wide-range protein standards (Novex) ranging from 2.5-200 kDa or the SeeBlue pre-stained standards (Novex) ranging from 4-250 kDa.

2.2.1 Techniques involved in the staining of SDS-PAGE gels

2.2.1.1 ***Coomassie staining and destaining.*** Coomassie staining was routinely used to visualise proteins analysed by SDS-PAGE by incubating gels in 0.1% (w/v) Coomassie Brilliant Blue R250/50% (v/v) methanol/7.5% (v/v) acetic acid for approximately 30 min. Gels were destained by washing gels in 7% (v/v) acetic acid/25% (v/v) methanol.

2.2.1.2 ***Silver staining of protein gels.*** Silver staining of SDS-PAGE gels was accomplished using the method described by Morrisey (1981). Gels were initially pre-fixed in 50% (v/v) methanol/10% (v/v) acetic acid for 30 min. This was followed by a second pre-fixative wash in a 5% (v/v) methanol/7% (v/v) acetic acid, again for 30 min. Fixation was achieved by a 30 min wash in 10% (v/v) glutaraldehyde (Sigma). The gel was then washed thoroughly in 5 x 1L of distilled water over a 2 h period and incubated in a 5 µg/ml DTT solution for 45 min. Without rinsing, the gel was then placed in a 0.1% (w/v) $AgNO_3$ solution, again for 45 min. After rinsing in distilled water for 30 sec, the gel was developed in 100 ml

3% (w/v) Na_2CO_3 /50 µl formaldehyde (BDH). Development was stopped by the addition of 5 ml 3M citric acid.

2.2.1.3 ***Copper chloride staining.*** Proteins which were to be excised from gels for electroelution were visualised by copper chloride staining (Lee *et al.,* 1987). Following electrophoresis, SDS-PAGE gels were rinsed in distilled water for 5 min, and agitated in a 0.3 M copper chloride (Sigma) solution for at least 5 min. On removal from the stain, gels were washed for 3 x 2 min in distilled water and then placed on a dark surface to highlight the negative staining of the proteins. Protein bands of interest were excised using a clean scalpel.

2.2.1.4 ***SYPRO™ Orange protein stain.*** SYPRO™ Orange (BioRad) was used as an alternative stain for the visualisation of proteins prior to electroelution, following SDS-PAGE analysis, gels were incubated in 7.5% (v/v) acetic acid/0.02% (v/v) SYPRO Orange, with gentle agitation for 30 min. They were then removed from the staining solution and rinsed in 7.5% (v/v) acetic acid for 30-60 seconds to remove excess stain from the gel surface. Protein bands were visualised by UV illumination using a UVtransilluminator (Hybaid). Gels images were documented using a UVP camera and video copy processor (Mitsubishi).

2.3 Protein Blotting

2.3.1 Semi-dry blotting

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) or PVDF membrane (Immobilon P, Millipore U.K. Ltd) using a semi-dry transfer apparatus (Milliblot

SDE system, Millipore) with a constant current of 1.2 mA/cm^2 of gel for 20 min. Membranes were pre-soaked in distilled water for 15 min prior to transfer. The transfer sandwich consisted of 1 filter paper soaked in 0.3 M Tris/10% (v/v) methanol (anode buffer 1), 3 filters soaked in 25 mM Tris/10% (v/v) methanol (anode buffer 2), the SDS-PAGE gel and membrane followed by 3 filters soaked in 25 mM Tris/10% (v/v) methanol/15 mM glycine (cathode buffer).

2.3.2 Wet blotting

Proteins analysed under non-denaturing conditions on SDS-PAGE were transferred onto PVDF membrane by wet blotting under mild conditions, in the absence of methanol (McCafferty *et al.,* 1995). The samples were transferred overnight at a constant 30 V in a BioRad Miniblot system (BioRad). The transfer buffer used was 25 mM Tris/200 mM glycine (pH 8.6). Following blotting, the membranes were processed as described in Section 2.3.3. Proteins to be analysed by N-terminal amino acid sequencing were transferred onto PVDF membranes, as described above, by wet blotting in the following transfer buffer: 50 mM sodium borate/20% (v/v) methanol/0.02% (v/v) 2-mercaptoethanol.

2.3.3 Antibody binding and detection

Nitrocellulose membranes were incubated for 2 min in a 10% (w/v) solution Ponceau S (Sigma) to identify tracks and confirm the success of protein transfer. Membranes were then washed for 3 x 1 min in a solution of 0.1% (w/v) Tween 20 (Sigma)/phosphate buffered saline (PBS), pH 7.4 (PBST), and incubated in the same solution for 30 min at room temperature to block non-specific protein adsorption to the membranes. Following the blocking procedure, the membranes

were rinsed (3 x 2 min) in PBST and incubated with optimally diluted primary antibody (see Table 1) for 1 h at room temperature, all subsequent washes and antibody dilutions were in PBST. The membranes were subsequently washed (3 x 5 min) and probed with optimally diluted (usually 1/1000 unless stated) alkaline phosphatase-conjugated rabbit anti-mouse IgG secondary antibody for 1 h at room temperature. Following final washing (3 x 5 min), proteins recognised by the primary antibody were visualised by incubating the filters in a substrate solution containing 100 mM Tris-HCl, pH 9.5/100 mM NaCl/50 mM Mg_2Cl_2/0.4 mg ml^{-1} nitro blue tetrazolium chloride (Boehringer)/0.2 mg ml^{-1} 5-bromo-4-chloro-3-indoyl-phosphate (Boehringer). Colour development was stopped by washing the membrane thoroughly in distilled water and the membranes were dried for storage.

Table 1: Antibodies

Antibody	Origin	Type	Specificity	Dilution	References
4/11	ascites	monoclonal	linear epitope in the VS2 region of *C. psittaci* S26/3 MOMP	1/1000	(McCafferty *et al.*, 1995)
A11	ascites	monoclonal	conformational *C. psittaci* MOMP	1/1000	(Andersen and Van Deusen, 1988)
E11	ascites	monoclonal	conformational *C. psittaci* MOMP	1/1000	(Andersen and Van Deusen, 1988)
C. psittaci '+' sheep sera *	sera	polyclonal	*C. psittaci* OEA epitopes	1/500	
181 (OEA serotype 1 specific) and 0040 (avian subspecies specific)	ascites	monoclonal	*C. psittaci* OMP90 proteins	1/1000	(Longbottom *et al.*, 1998a; Vretou *et al.*, 1996)
13/4	ascites	monoclonal	*Chlamydia* LPS	1/1000	
Cpn 1	sera	polyclonal	recombinant *C. pneumoniae* MOMP	1/500	
anti-*C.pneumoniae* antibody	ascites	monoclonal	EBs of *C. pneumoniae*	1/1000	Dako M 6600

* pool of post-abortion sera from animals experimentally infected with OEA subtype *C. psittaci*

2.3.4 Techniques involved in the staining of membranes

2.3.4.1 ***Protogold staining of PVDF membranes:*** Following overnight transfer of protein onto PVDF membrane, as described in Section 2.3.2, membranes were washed in PBS for 2 x 5 min and incubated in PBS containing 0.3% (v/v) Tween 20 (Sigma) at 37°C for 30 min, to block unoccupied binding sites. Membranes were then washed 3 x 5 min in PBS, again containing 0.5% (v/v) Tween 20, and 3 x 1 min with distilled water to remove any buffered salts. These washing steps dramatically reduce background staining and were followed by the incubation of the membranes in Protogold, a stabilised colloidal gold solution (British Bio-Cell, Cardiff, UK). Optimum staining times varied between 2-4 h, depending on the concentrations of protein involved. After staining, membranes were washed in distilled water and dried for storage.

2.3.4.2 ***Coomassie staining of PVDF membranes:*** The staining of protein bands on PVDF membranes for excision and N-terminal sequencing was achieved using an adapted Coomassie Blue protocol. PVDF membranes were rinsed (3 x 2 min) with distilled water and saturated with 100% (v/v) methanol for 20 seconds. Membranes were then stained in 40% (v/v) methanol/1% (v/v) acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma). Once the required staining was achieved, usually within one min, the membrane was destained in 50% (v/v) methanol and finally rinsed with distilled water. Protein bands of interest were excised using a clean scalpel and finally sequenced by the Microchemical Facility, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK.

2.4 Purification of MOMP

2.4.1 Differential solubilisation of MOMP

MOMP was solubilised from sarkosyl (sodium lauroyl sarcosinate) (Sigma) extracted preparations of the OEA isolate of *C. psittaci*, as described previously (McCafferty *et al.*, 1995). Briefly, 20 mg of EBs/RBs from tissue culture harvest were suspended in 2% (w/v) sarkosyl and incubated at 37°C for 1 h. After centrifugation for 30 min at 100,000 x g, the pellet was resuspended in 2% (w/v) sarcosyl containing 10 mM DTT (Sigma) and incubated at 37°C, again for 1 h. Finally, the pellet was resuspended in 2% (w/v) OG (Boehringer) containing 10 mM DTT, incubated for 2 h at 37°C and centrifuged for 30 min at 100,000 x g. The supernatant, which contained solubilised MOMP, was collected and stored at -20°C.

Solubilisation of MOMP was attempted using a variety of detergents to ascertain if these could improve the purity of solubilised MOMP, in comparison to the OG/DTT solubilised sample. The pellet resulting from incubation in sarcosyl/DTT was re-suspended in the following detergents in the presence of 10 mM DTT and incubated for 2 h at 37°C: 2% (w/v) octyl polyoxoethylene (POE) (Bachem Feinchemikalien); 2% (w/v) sodium deoxycholate (Sigma); 2% (w/v) sodium dodecyl sulphate (Sigma) and 2% (w/v) Hecameg (BDH). Following this incubation, samples were centrifuged for 30 min at 100,000 x g. The resulting pellets and supernatants were collected and analysed by SDS-PAGE and Western blotting.

2.4.2 Sucrose density centrifugation

0.1 ml OG/DTT solubilised MOMP was layered onto a 5-20% (w/v) linear

gradient of sucrose (BDH). Gradients were prepared using the method described by McCafferty *et al.* (1995) and left to diffuse at room temperature for 5 h. After sample loading, gradients were centrifuged at a constant temperature of 20°C in a Beckman SW55 rotor for 16 h at 130,000 x g. 1 ml samples collected in a fraction collector using a peristaltic pump (P-3, Pharmacia), were analysed by SDS-PAGE. An adaptation of this protocol, where a 60% (w/v) cushion of the centrifugation medium Nycodenz (Nyegaard Diagnostica, Oslo, Norway) was added to the base of the sucrose gradient, was also carried out in an attempt to stop MOMP forming an insoluble pellet.

2.4.3 Preparative isoelectric focusing

Preparative isoelectric focusing was achieved using the MinipHor protein fractionator (Rainin Protein Technologies, Inc). Focusing was carried out according to manufacturers' instructions. Briefly, the system was filled with a 1% (v/v) ampholyte (0.2% pH 3-10 and 0.8% pH 5-7)/10% (v/v) glycerol solution. The ampholytes were pre-focused at a constant 1000 V for 45 min before protein samples were loaded. 1-3 ml of protein sample were loaded directly onto the pre-formed gradient and focused at 1000 V for 30 min followed by a further 10 min at 500 V. Fractions were collected in 0.8 ml aliquots and analysed by SDS-PAGE.

2.4.4 Electroelution

The model 422 Electro-Eluter (BioRad) was used to recover proteins from SDS-PAGE gel slices. Excised protein bands were washed 3 x 10 min in 0.25 M Tris, pH 9.3 containing 0.25 M EDTA (Sigma). Gel slices were equilibrated prior to electroelution in elution buffer (25 mM Tris/192 mM glycine/0.1% (w/v) SDS,

pH8.3) at room temperature for 1 h. Electroelution was carried out according to manufacturers' instructions at a constant current of 8-10 mA/glass elution tube for at least 3 h. Eluted proteins were collected and stored at -20°C.

2.4.5 Hydroxyapatite chromatography

MOMP was purified by non-denaturing hydroxyapatite chromatography, primarily using the technique described by Caldwell *et al.* (1981) with some minor modifications. Briefly, 15-20 mg of EBs/RBs from tissue culture harvest were suspended in 2% (w/v) sarkosyl (Sigma) and incubated for 1 h at 37°C. Following centrifugation for 30 min at 100,000 x g the pellet was resuspended in 2% (w/v) SDS/PBS/1 mM DTT and incubated for 1 h at 37°C. 7 mg of the MOMP-enriched SDS extract was equilibrated in 0.01 M sodium phosphate, pH 6.4/1 mM DTT/0.1% (w/v) SDS (column equilibration buffer) and fractionated by hydroxyapatite chromatography in the continued presence of SDS using the technique of Moss and Rosenblum (1972). The MOMP-enriched sample was loaded onto a pre-equilibrated hydroxyapatite column (BioRad) (5 cm x 1 cm) using a peristaltic pump at a rate not exceeding 0.5 ml/min and the column was then washed with equilibration buffer until the absorbance at 280 nm was below 0.05. Fractions were eluted using a linear gradient of 0.1-0.6 M sodium phosphate, pH 6.4 containing 1 mM DTT and 0.1% (w/v) SDS at a rate not exceeding 0.1 ml/min and the absorbance at 280 nm monitored. Those fractions showing increased absorbance at 280 nm were analysed by SDS-PAGE and by immunoblotting using the MOMP-specific monoclonal antibody 4/11 (Table 1).

2.5 Protein estimation

Protein concentration was determined by gel densitometry using the BioRad GS-670 imaging densitometer and by using the bicinchoninic acid protein assay (BCA; Pierce). Bovine serum albumin (BSA) was used as a standard.

2.6 Structural analysis of MOMP

2.6.1 Secondary structure prediction

Secondary structure predictions of MOMP from *C. psittaci* OEA subtype were performed using DNAStar DNA/protein sequence analysis software (DNASTAR, Inc.). The secondary structure prediction alogarithms applied to the MOMP sequence were Chou-Fasman (Chou and Fasman, 1978) and Garnier-Robson (Garnier *et al.,* 1978).

2.6.2 Circular dichroism

CD analysis of OG/DTT-solubilised MOMP and hydroxyapatite-purified MOMP was performed on a Jasco J-600 spectrophotometer with a pathlength of 0.02cm, averaging 16 scans between wavelengths of 190 nm and 260 nm for each sample. The spectrophotometer was blanked using buffer alone. Secondary structure estimations were obtained using the CONTIN procedure of Provencher and Glockner (1981), which essentially compares the spectrum to a database of spectra and structure.

2.7 Isoelectric focusing

2.7.1 2-D gel electrophoresis

2-D gel electrophoresis of OG/DTT solubilised MOMP was performed using the Mini Protean II 2-D electrophoresis cell (BioRad). Focusing was carried out according to the manufacturers' instructions. Briefly, the first dimension polyacrylamide tube gels containing a 1.6% (v/v) pH 5- 7 and a 0.4% (v/v) pH 3-10 ampholyte mix (BioRad) were cast in capillary tubes. Samples were equilibrated for 30 min at room temperature in first dimension sample buffer which also contained a 1.6% (v/v) pH 5-7 and a 0.4% (v/v) pH 3-10 ampholyte mix (9.5 M urea/2% (v/v) Triton X-100 (Sigma)/5% (v/v) 2-mercaptoethanol). The ampholytes within the tube gels were focused, prior to the loading of the samples, by applying 200 V for 10 min, 300 V for 15 min and 400 V for 15 min. Following loading, samples were focused at a constant of 500 V for 10 min and 750 V for 3.5 h. After first dimension focusing, the tube gels were flushed from the capillary tubing and equilibrated in second dimension SDS sample equilibration buffer (0.0625 M Tris-HCl, pH 6.8/2.3% (w/v) SDS/5% (v/v) 2-mercaptoethanol/10% (w/v) glycerol/0.0025% (w/v) bromophenol blue) for 30 min. Tube gels were then placed on the top of 12.5% gels for SDS-PAGE in the second dimension (Section 2.2). The resulting 2D-gels were silver stained (Section 2.2.1.2).

2.7.2 Isoelectric focusing

Isoelectric focusing was carried out using the XCell II Mini-Cell electrophoresis system (Novex) in conjunction with precast 5% (w/v) polyacrylamide, non-denaturing IEF gels (Novex). Gels with an ampholyte range of pH 3-10 and pH 5-7 were run using the buffering system recommended by the

manufacturer. Briefly, pH 3-7 IEF gels were run with a cathode buffer of 0.6% (w/v) lysine (Sigma) and anode buffer of 0.08% (w/v) phosphoric acid, while pH 3-10 gels were separated in 0.35% (w/v) arginine/0.3% lysine buffer (cathode) and 0.08% phosphoric acid buffer (anode). Following sample loading, gels were run at 100 V constant voltage for 1 h, 200 V for 1 h and 500 V for 30 min. The approximate current during this time was 5 mA/gel. The gels were fixed on completion of focusing in a 12% (v/v) trichloroacetic acid solution for 30 min and then silver stained as described in Section 2.2.1.2. Alternatively, the proteins were transferred onto PVDF membrane using an adapted Western blotting protocol. Protein transfer was carried out using the BioRad Miniblot system (BioRad) and a 0.7% (v/v) acetic acid (pH 3.0) transfer buffer at 10 V constant voltage for 1 h.

2.8 Molecular biology techniques

2.8.1 Agarose gel electrophoresis

DNA was analysed by conventional agarose gel electrophoresis. Agarose concentrations ranging from 0.8-2.0% (w/v) were prepared in TAE electrophoresis buffer (40 mM Tris-acetate, pH8.0/1 mM EDTA) in the presence of 0.5 μg of ethidium bromide per ml. Gels were run in TAE buffer at a constant voltage of 60 volts. A range of molecular weight standards were included in each run, usually a 1 kb DNA Step ladder or ϕX174/Hae III markers (both from Gibco). DNA fragments were visualised by UV illumination using a Hybaid transilluminator. Gel documentation was provided by UVP camera and video copy processor (Mitsubishi).

2.8.2 *C. psittaci* OEA tMOMP construct

The *C. psittaci* MOMP construct contained the coding region for a truncated form of the protein, hence the name "t"MOMP (Hering *et al.*, 1998). The tMOMP construct consisted of the coding region of *C. psittaci* OEA isolate MOMP, beginning at the first natural methionine, cloned into pET-22b (+). On induction, the construct produced a shortened protein lacking the first 16 amino acids of MOMP. Expression of the tMOMP construct in this study was as described for the *C. pneumoniae* recombinant MOMP construct in Section 2.8.12.

2.8.3 *C. pneumoniae* tMOMP construct

2.8.3.1 Polymerase chain reaction (PCR)

The cloning and expression of the *C. pneumoniae* recombinant MOMP, described in the following sections, was largely based on that of the *C. psittaci* tMOMP construct (Herring *et al.*, 1998)(Section 2.8.2). The *C. pneumoniae* MOMP gene was amplified by PCR from *C. pneumoniae* strain IOL207 genomic DNA (kind gift of the late J. Treharne). The primer pairs were:

5′-ATCGATGGC**CATATG**TGGGAAGGTG-3′ plus

5′-GGGC**GAATT**CTTATGCGAATGGAT-3′ and

5′-TGATGGT**CATATG**TGGGAAGGTGCTGCAGG-3′ plus

5′-A**GCGGCCGC**TCAGAATCGAACT-3′ for *C. psittaci* (GenBank Accession Number X51859) and *C. pneumoniae* (GenBank Accession Number M69230), respectively. Primers were designed using the program Oligo. PCR reactions were carried using the Expand Long Template PCR System (Boehringer). 50 µl final reaction volumes containing template DNA, 300 nM upstream and downstream

primers, 350 µM dNTPs (Boehringer), PCR buffer (including 1.75 mM Mg^{2+}) and *Pwo/Taq* polymerase. Reactions were overlayed with sterile mineral oil (Sigma) to prevent evaporation. PCR conditions were as follows: a 2 min denaturation at 94°C for 1 cycle; followed by 45 sec denaturation at 94°C, 30 sec annealing at 44°C and 40 sec extension at 68°C for 30 cycles; and a final 7 min extension at 68°C for 1 cycle.

2.8.3.2 Purification of DNA fragments from agarose gels

PCR products, utilised for cloning, were fractioned by electrophoresis in low melting point agarose (Boehringer). Bands of interest were excised and purified from the agarose gel slice using a QIAquick gel extraction kit (Qiagen), according to the manufacturers' instructions and based on the protocol of Vogelstein and Gillespie (1979).

2.8.3.3 Ligation of PCR products into the pGEM-T cloning vector

PCR products were cloned into the T-vector, pGEM-T (Promega). The T-vector system takes advantage of the non-template dependent addition of a single deoxyadenosine to the 3′ end of PCR products by thermostable *Taq* which can be ligated to the single 3′-T overhangs of the vector. The gel-purified PCR products were ligated into pGEM-T using the T4 DNA ligase enzyme (Promega) in a total reaction volume of 10 µl : T4 DNA ligase buffer (30 mM Tris-HCl, pH 7.5/10 mM $MgCl_2$/10 mM DTT / 1 mM ATP), containing 50 ng of vector, insert DNA [at an insert:vector molar ratio of 3:1 or 1:1] and 1 Weiss Unit/µl T4 DNA ligase (Promega). Concentrations of vector and insert DNA were estimated by comparison

with DNA molecular weight markers of known concentration. The molar mass ratio for DNA molecules was estimated using the following formula: [ng vector x insert size (kb)/ vector size (kb)] x molar ratio of insert:vector = ng of insert required]. Following overnight incubation at 6 °C, the ligation mixes were transformed into chemically competent XL-1 blue cells (Section 2.8.9).

2.8.3.4 Dephosphorylation of restriction endonuclease-digested pET-22b (+)

The pET-22b (Novagen) expression vector was digested with restriction enzymes, *Not*I and *Nde*I (Promega), in preparation for ligation with the *C. pneumoniae* MOMP DNA fragment excised from pGEM-T (Section 2.8.3.5). The 5′ ends of the linearised vector were dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (Promega). Routinely, 1U CIAP was added to the RE digestion reaction mix in CIAP reaction buffer (Promega) and incubated for 1h at 37°C. The reaction was stopped by adding 5 mM EDTA/0.5% (w/v) SDS, then adding proteinase K to a final concentration of 100 µg/ml and finally incubating at 56 °C for 30 min. The DNA was extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol, vortexing for 1 min, centrifuging at 9,000 x g for 5 min and removing the upper aqueous layer. The DNA was precipitated by adding ammonium acetate (freshly prepared) (final concentration of 2.5 M), 3 volumes of 100% (v/v) ethanol and placing on ice for 30 min. After centrifugation at 9,000 x g for 30 min, the pellet was washed with 0.5 ml 70% (v/v) ethanol. Following centrifugation, again at 9,000 x g for 15 min, the pellet was air dried and solubilised in nuclease-free water.

2.8.3.5 Ligation into pET-22b (+) expression vector

The *C. pneumoniae* MOMP DNA fragment was excised from pGEM-T using restriction enzymes *Nde*I and *Not*I and ligated into the pET-22b expression vector, as described in Section 2.8.3.3. The ligation mix was initially transformed (Section 2.8.7.3) into competent (Section 2.8.3.6) XL1-Blue cells (Stratagene), which lacks the gene for T7 RNA polymerase to allow examination of the construct sequences. The pET construct was subsequently transformed into expression host BL21 (DE3), which contains the T7 RNA gene, and was induced as described in Section 2.8.4.

2.8.3.6 Preparation of competent cells

Competent BL21 (λ DE3) and Epicurian Coli XL-1 Blue cells were prepared using the following protocol. Glycerol stocks were streaked out onto agar plates containing the appropriate antibiotic (tetracycline for XL-1 Blue and none for BL21 cells) and incubated at 37°C overnight. One colony was picked from each plate and used to inoculate 10 ml of L-broth containing the appropriate antibiotic which was then incubated in a shaking incubator overnight at 37°C. 1 ml of these overnight cultures was added to 100 ml of L-broth plus antibiotic, pre-warmed to 37°C. Cultures were incubated in a shaking incubator at 37°C until the absorbance at 600 nm, as measured on a Beckman DU-600 spectrophotometer, reached 0.4. After cooling on ice, cultures were centrifuged at 1500 x g for 10 min. The resulting pellet was resuspended in 0.1x its original volume in ice cold transformation and storage solution (TSS) (10% (w/v) polyethylene glycol (PEG) 8000 (BDH)/20 mM $MgCl_2$/ 5% (v/v) DMSO (Sigma)/L broth). The cells were stored at -70°C in 0.3-

0.5 ml aliquots.

2.8.3.7 Transformation of competent cells

In this study, PCR inserts were ligated into pGEM-T and transformed into XL-1 Blue for the purpose of propagation and storage. Restriction endonuclease excised inserts were ligated into pET-22b (+) and transformed into XL-1 Blue for the purpose of propagation and to allow examination of construct sequences, and then transformed into BL21 (λDE3) for transient expression.

50 µl of competent cells (Section 2.8.3.6), thawed on ice, were added to 2 µl ligation mixture (Section 2.8.3.3 and 2.8.3.5) and left on ice for 20 min. Cells were transformed by heat shock at 42°C for exactly 45 seconds. Following heat shock, transformation mixes were placed on ice for a further 2 min before 0.5 ml SOC medium (2% (w/v) tryptone/0.5% (w/v) yeast extract/10 mM NaCl/2.5 mM KCl/10 mM $MgCl_2$/10 mM $MgSO_4$/20mM glucose) was added to each reaction. After incubation for 1 h at 37°C, 50-200 µl of the reaction mixes were plated out on L-agar plates containing ampicillin (50 µg/ml), tetracycline (30 µg/ml), X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Sigma) (80 µg/ml) and IPTG (isopropyl-thiogalactoside) (Sigma) (0.5 mM). IPTG and X-Gal were added for blue white colour selection of recombinant colonies when plating out pGEM-T/XL1-Blue transformants (No IPTG or X-Gal was added when plating out pET transformants). pGEM-T contains a multiple cloning site in the α-peptide coding region of the β-galactosidase enzyme. When appropriate strains of *E. coli* are used, insertional inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening on plates containing the lactose analogue X-gal and

an inducer of the *lac* operon, IPTG. Non-recombinant colonies synthesise β-galactosidase, which breaks down X-gal to a blue product resulting in blue colonies, while the recombinant colonies synthesise an inactive form of the enzyme and remain white. The plates were incubated at 37°C overnight. White colonies were picked and inoculated into 10 ml of L-broth containing ampicillin and tetracycline when selecting for XL-1 Blue recombinant colonies and ampicillin for BL21 (DE3) colonies. The broths are then incubated in a shaking incubator at 37°C overnight.

2.8.3.8 Preparation of plasmid DNA

10 ml of overnight bacterial culture was centrifuged at 1500 x g for 10 min at room temperature. Plasmid DNA was purified using a QIAprep^{TM} kit (Qiagen), according to manufacturers' guidelines. The QIAprep kit protocol is based on the modified alkaline lysis method of Birnboim and Doly (1979) where bacterial cells are lysed in NaOH/SDS lysis buffer. After neutralisation, chromosomal DNA is precipitated by the addition of guanidine-HCl, the precipitate is pelleted by centrifugation at 9,000 x g, the supernatant containing plasmid DNA is removed and is adsorbed onto a silica-based gel by centrifugation in the presence of high salt and, after washing, the plasmid DNA is eluted using TE buffer (10 mM Tris, pH8.0/1 mM EDTA).

2.8.3.9 Restriction endonuclease digestion of DNA

Restriction enzymes were purchased from Boehringer and MBI Fermantas and used with appropriate incubation buffers, as recommended by the manufacturer. Routinely, 10 U enzyme/μg DNA was used per reaction.

2.8.4 Inclusion body preparation

BL21 (λDE3) cells transformed with recombinant MOMP from either OEA *C. psittaci* or *C. pneumoniae*, in expression vector pET-22b (+) were picked from agar plates and used to inoculate 10 ml L-broth containing ampicillin (100μg/ml). Cultures were grown for approximately 2 h at 37°C and then centrifuged at 1500 x g for 10 min. The supernatant was discarded and 400 ml of fresh L-broth containing ampicillin (100μg/ml) was used to resuspend the resulting pellet. Again the culture was incubated at 37°C until its absorbance at 600 nm, as measured on a Beckman DU-600 spectrophotometer, reached 0.6-0.7. At this point, 1.5 ml of the culture was removed and used as the 0 h control. IPTG (Boehringer) was added to the remaining culture to a final concentration of 1 mM. The culture was incubated for a further 3-3.5 h at 37°C, then centrifuged at 4,000 x g to harvest the cells. The cells were washed in 100 ml PBS and centrifuged at 4,000 x g for 10 min. The resulting pellet was resuspended in 100 ml TESL (0.25 M Tris, pH 8/1 M sucrose/50 mM EDTA/24 mg ml^{-1} lysozyme (Sigma)) and incubated at room temperature for 15 min before centrifugation at 12,000 x g. The pellet was then washed in 100 ml TES (0.25 M Tris, pH 8/1 M sucrose/50 mM EDTA), centrifuged at 12,000 x g and the resulting pellet was resuspended in 80 ml TE buffer. This suspension was sonicated for 6 x 15 sec with 1 min intervals between bursts and then centrifuged at 7,000 x g for 15 min. This pellet was then washed twice in 80 ml PBS, again centrifuged at 7,000 x g before the resulting pellet was resuspended in 1ml PBS.

Inclusion body-expressed MOMP was analysed by SDS-PAGE and prepared for planar lipid bilayer incorporation (Section 2.10.1). 50 μl of inclusion body protein, suspended in PBS, was centrifuged at 9,000 x g for 5 min. The supernatant

was removed, the resulting pellet suspended in 2% (w/v) octyl glucoside/10 mM DTT and incubated at 37°C for 1h. Following incubation the sample was again centrifuged at 9,000 x g for 5 min. The resulting supernatant was removed and stored at -20°C, in 5 µl aliquots until use, for bilayer incorporation

2.8.5 *In vitro* cell-free coupled transcription/translation

The *E. coli* T7 S30 extract system for circular DNA (Promega) was used for the coupled transcription/translation of a truncated MOMP construct, tMOMP. This system simplifies the transcription/translation of DNA sequences cloned in plasmids or λ vectors containing a T7 promoter by providing an extract containing a T7 polymerase for transcription and all the necessary components for translation. This kit is prepared using a modification of the method described by Yang *et al.* (1980).

Briefly, tMOMP DNA (<4 µg), prepared using a QIAprep^{TM} kit as described in Section 2.8.3.8, was incubated with an amino acid mixture, S30 pre-mix without amino acids, T7 S30 extract and nuclease-free water in a final reaction volume of 50 µl according to manufacturers' instructions (Promega). Reactions were incubated at 37°C for 2 h and then placed on ice for 5 min. The PinPoint™ vector provided by the manufacturer was used as a control. Translated proteins were detected using radioactive labelling by adding [^{35}S] L-methionine (1,2000Ci/mmole specific activity and concentration of 15 mCi/ml) to the reaction mixture. Resulting proteins were analysed by SDS-PAGE, blotted onto nitrocellulose membrane, which was used to expose x-ray film (Fuji) and developed using a X-ograph Compact x 2 imager (X-ograph Imaging Systems, Malmesbury, U.K.).

2.9 Recombinant *C. pneumoniae* MOMP antibody production

2.9.1 Antigen preparation

The aqueous phase of the *C. pneumoniae* recombinant MOMP vaccine was prepared as follows: Glutaraldehyde was added to the antigen suspension to a final concentration of 0.01% (v/v), mixed well by vortexing and left at room temperature for 30 min, with further vortexing at 5 min intervals for the first 15 min. The inactivation of the antigen by glutaraldehyde was quenched by the addition of sterile filtered glycine to a final concentration of 10 mM. This reaction mixture was mixed thoroughly then incubated at 4°C for 5 min. Finally, Thimerosal (Sigma) was added to this aqueous phase of the antigen to a final concentration of 0.01% (v/v).

The aqueous phase of the antigen was mixed 70/30 (adjuvant/aqueous phase) with the adjuvant Montanide ISA 773 (Seppic, Paris, France). Initially the adjuvant was homogenised using the Ultra Turrax T25 homogeniser, with a S25 KG probe, at 8,000 rpm. The aqueous phase was added to the adjuvant in 2-3 ml aliquots while homogenising. To avoid overheating of the antigen mixture, homogenisation was stopped every 10-20 sec for 10 sec. Once the entire aqueous phase had been added to the adjuvant, the suspension was homogenised in 10 sec bursts (with 10 sec pauses between bursts), through 6 cycles. This was repeated with homogenisation at 9,000 rpm and finally 13,500 rpm, for 3 cycles. Sterility of the emulsified antigen was checked by streaking out on agar plates (in the absence of antibiotics), incubating at 37°C and monitoring for bacterial growth. The antigen was stored at 4°C until inoculation.

2.9.2 Antiserum production

Animals (3 x rabbits, plus 3 x guinea pigs) were injected subcutaneously with purified recombinant *C. pneumoniae* MOMP (Section 2.8.4) (100 µg per dose) prepared with adjuvant Montanide ISA 773. Animals were immunised twice, 3 weeks apart, and each dose was administered at 2 different sites (inoculation volume did not exceed 0.25 ml). Intra venous blood samples were taken before the first inoculation. At 3 week post final vaccination animals were anaesthetised using Halothane with NO_2 and O_2 and bled out. Sera was prepared by allowing samples to clot overnight at 4°C before centrifugation at 3,000 x g for 20 min. The serum was then removed and stored in aliquots at -20°C.

2.10 Planar lipid bilayer reconstitution and channel analysis

2.10.1 MOMP bilayer reconstitution

MOMP was incorporated into 0.3 mm diameter lipid bilayers cast at room temperature from a 30 mg/ml decane suspension of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, AL, USA) using equipment and techniques similar to those described by Williams (1995) and depicted in Figure 1.1. Briefly, a small amount of lipid (~1 µl) was drawn across a 300µm hole in a polystyrene partition separating two solution-filled chambers (600µl) which were designated *"cis"* and *"trans"* . Bilayers formed spontaneously as observed by monitoring the increase in membrane capacitance which accompanied thinning. The bilayer capacitance was measured using a triangle waveform pulse of 100 Hz at 100 mV. The *"cis"* side of the bilayer was voltage clamped at a potential relative to the *"trans"* side using a Biologic RK-300 patch clamp amplifier (Intracel, Royston, U.K.). The relative potential applied

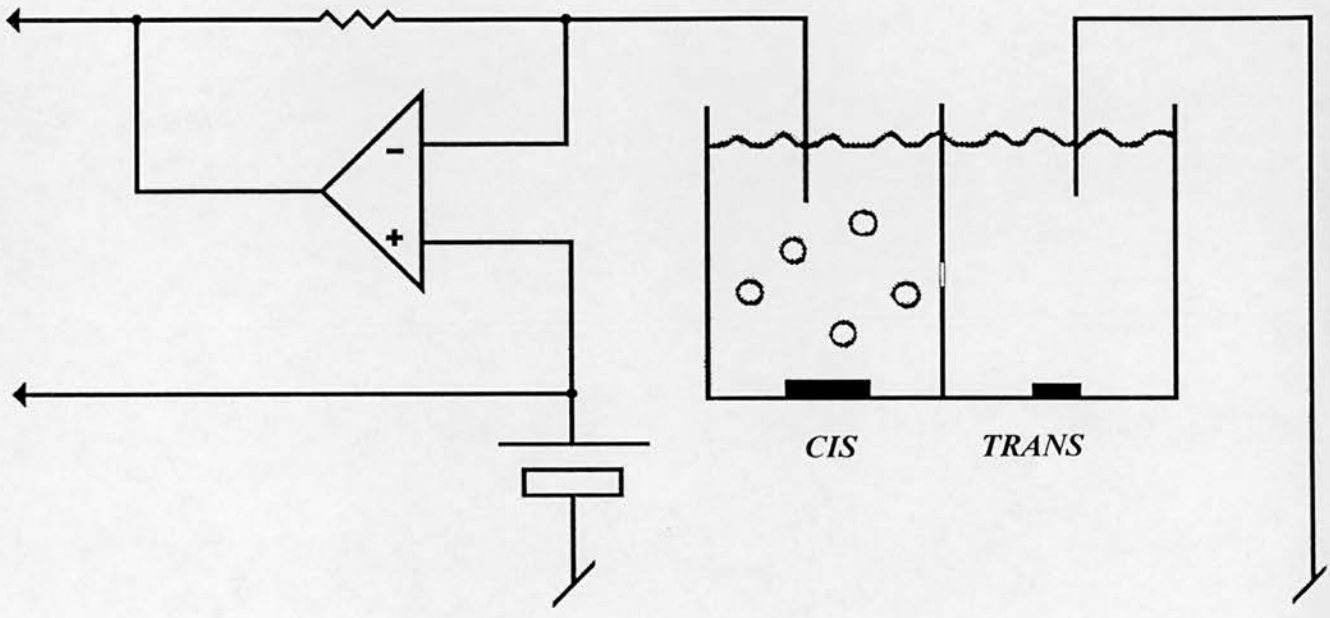


Figure 2.1 Schematic representation of the planar lipid bilayer set-up.

across the bilayer was termed the "holding potential" or "voltage clamp potential". Transmembrane currents were low-pass filtered (10 kHz, 8-pole Bessel filter) and digitally recorded. All the bilayers used had a conductance of <10 pS and a capacitance of >250 pS. Bilayers of this size have a relatively large capacitance. When large changes are made to the holding potential (eg: switching from 0 mV to +/- 80 mV) the bilayer momentarily becomes charged, and the charge then dissipates almost immediately to give rise to an exponentially-decaying current transient. This is referred to as the "bilayer capacitative current transient" and is superimposed on channel recordings made immediately after large changes in the holding potential, giving them a characteristic "curved" appearance.

To incorporate channels, purified detergent-solubilised native MOMP (Section 2.4.1), or detergent-solubilised recombinant MOMP (Section 2.8.4), was added to the *cis* chamber to a final concentration of 1 ng/ml, in the presence of a salt gradient, 250 mM KCl *cis* versus 50 mM KCl *trans* (buffered with 10 mM Tris-HCl, pH 7.4). The solutions bathing the bilayer were changed by perfusion (at least 10 volumes) as required. Opening and closing of the ion channel gave rise to square shaped pulses of current which are termed "unit currents". Membrane current and holding (voltage clamp) potentials are displayed according to a standard convention, quoting the holding (clamp) potential in the *cis* chamber, with positive transmembrane (upgoing) currents representing a net flux of cations flowing *cis* to *trans*, or a net flux of anions flowing in the opposite direction.

2.10.2 Channel analysis

Channel recordings were post-filtered (see Figure Legends) to reduce high frequency noise and analysed using the program pClamp 6 (Axon Instruments, CA,

USA). In the presence of a salt gradient either side of the bilayer, the reversal potential is defined as the holding potential that exactly balances the tendency for ions to diffuse down their chemical concentration gradient. Relative ionic permeabilities were calculated from measured reversal potentials using appropriate forms of the Goldman-Hodgkin-Katz (GHK) equation. Briefly, with the same monovalent salt in both chambers.

$$Er = -RT/zF.\ln(P_C[C]_t + P_A[A]_c/P_C[C]_c + P_A[A]_t)$$

where Er is the reversal potential, P is permeability, A and C are the anion and cation, and *c* and *t* represent *cis* and *trans*, respectively. R is the gas constant, T is room temperature, z is valency and F is the Faraday constant. Concentrations were corrected for ionic activities using activity coefficients obtained from standard tables.

2.10.3 Additions to bilayer solutions

2.10.3.1 ***Monoclonal antibodies.*** Antibodies (Table 1) (final dilution of 1/1000) in 50 mM KCl, 10 mM Tris-HCl, pH 7.4 and 1 mg/ml BSA were added to both the *cis* and *trans* chambers. MOMP channel activity, in the presence of antibodies, was observed and recorded for a minimum of 15 min.

2.10.3.2 ***Oxidising agents.*** 10-500 mM Cu^{2+}-phenanthroline (Boehringer), hydrogen peroxide (Sigma) or oxidised glutathione (Sigma) in 50 mM KCl, 10 mM Tris-HCl, pH 7.4 were added to the *cis* and *trans* chambers. Channel activity in bilayers bathed in these buffers were monitored over a period of at least 30 min.

2.10.3.3 ***ATP.*** ATP was added to the bilayer in the form of 10 mM Na^{+}-ATP

(Boehringer), 10 mM Tris-HCl, pH 7.4 in both the *cis* and the *trans* chambers. More concentrated solutions were achieved by the addition of Na^+-ATP to the chambers from a concentrated stock solution. For multivalent ATP^{4-} ion in the presence of Na^+, the theoretical Er for a ATP^{4-} selective channel was estimated from the Nerst equation. The *activity* ratio of 100 mM Na^+-ATP: 10 mM Na^+-ATP was measured using the Na^+ ionophore gramicidin (Gennis, 1989).

2.11 Heparin/Heparan sulphate studies

2.11.1 Heparin titration

McCoy cell monolayers were established in 7 ml vials, containing glass coverslips, by seeding at 2 x 10^5 cells/ml in cell culture medium (Section 2.1) and incubating at 37°C for 24 h. Cell culture medium was then aspirated and cell monolayers were incubated in fresh medium containing a 10^{-4} dilution of *C. psittaci* inoculum and 0-500 U/ml of heparin (sodium salt from ovine intestinal mucosa) (Sigma) for 1 h at 37°C. Following this incubation, cell culture medium was again aspirated, cell monolayers were washed with 3 x 1 ml PBS and incubated with fresh culture medium for 3 days at 37°C. Infected cell monolayers on plastic coverslips were then removed from culture medium, fixed and stained using the Giemsa method (Section 2.11.3). Coverslips were then mounted on glass slides and counted to determine the number of inclusion bodies. Each experiment was carried out in triplicate.

2.11.2 Heparitinase treatment of *C. psittaci* OEA isolate EBs

5 x 10^5 inclusion forming units/ml of the OEA isolate of *C. psittaci* were

incubated in 0-2.0 U/ml heparitinase III (Sigma) in PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 1 h at 37°C. Following heparitinase treatment the chlamydiae were washed by centrifugation (9000 x g, 10 min) in 2 x 1 ml PBS and resuspended in 1 ml of cell culture medium. McCoy cell monolayers in cell culture medium, as described above, were incubated at 37°C for 3 days. Infected cell monolayers, on glass coverslips, were harvested, fixed, stained and counted as described in Section 2.11.3.

2.11.3 Fixing and Giemsa staining of infected cell coverslips

Infected cell monolayers on plastic coverslips were removed from culture medium and fixed by incubation in 100% (v/v) methanol (Fisons) for at least 5 min. The coverslips were then immersed in 5% (v/v) Giemsa stain (BDH) for 20 min. The Giemsa-stained coverslips were then washed for approximately 30 sec in water. Following washing, the coverslips were immersed for 30 sec in the following solutions: 100% (v/v) acetone (Fisons); 65% (v/v) acetone/35% (v/v) xylene (Fisons); 50% (v/v) acetone/50% (v/v) xylene; 35% (v/v) acetone/65% (v/v) xylene; and 100% (v/v) xylene. Stained coverslips were mounted onto glass slides using DPX mountant (BDH) and left to dry at room temperature for 1h. Inclusion bodies present on infected coverslips were counted using bright field illumination at a magnification of x 400.

2.11.4 Binding of [^{35}S] heparin to non-denatured MOMP

MOMP solubilised in OG-DTT was run on a SDS-PAGE gel under non-denaturing conditions, as described in Section 2.2. Protein was transferred onto

nitrocellulose membranes. Non-specific binding sites on membranes were blocked by a 3 h incubation in PBS containing one of the following: 5% marvel/0.05% Tween 20; 0.5% Tween 20/5% marvel; 0.1% gelatin/0.5% Tween 20; 0.1% gelatin/0.05% Tween 20; and 1% polyethylene glycol (PEG). Membranes were washed for 2 h in PBS with changes of wash buffer every 10 min and then incubated in PBS containing 30mCi of [*N* - Sulphonate-^{35}S]Heparin (Amersham) for 2 h. Following this incubation, membranes were washed for 2 h in PBS, air-dried and exposed to x-ray film (Fuji) for 3-12 h and developed using a X-ograph Compact imager.

CHAPTER THREE

RESULTS:

PURIFICATION AND STRUCTURAL ANALYSIS OF MOMP

3.1 Introduction

Despite many years of intensive study, the lack of structural information leaves unanswered many questions as to how MOMP may fulfil its suggested diverse functions (Bavoil *et al.,* 1984; Su *et al.,* 1990). Structural studies are hampered firstly by the difficulty of growing chlamydiae in bulk and subsequently by problems with purifying and solubilising a protein which is both highly disulphide bond cross-linked (Newhall, 1987) and normally resides in a hydrophobic environment. These factors have made it necessary to rely on analytical techniques that require relatively small quantities of protein. In addition, due to the extensive disulphide bond cross-linking present in MOMP, expression of recombinant protein which refolds to achieve native conformation has been almost impossible. The inability to refold recombinant MOMP, and the difficulties in obtaining large quantities of purified native protein, suggest that x-ray crystallography of MOMP is, at present, an unrealistic goal. However, reliable preliminary structural information can be obtained using techniques, such as circular dichroism, which require far less protein. Structural information may lead to the establishment of a MOMP structural model which, in turn, will aid the development of future MOMP-based vaccines.

The two primary goals of this project were (1) to gain structural information for MOMP, using techniques such as CD and (2) to directly test the MOMP porin hypothesis (Section 1.8.6) at the molecular level using the planar lipid bilayer system. In both these cases maintaining the structural integrity of purified MOMP was essential. Taking this into consideration suitable methods for purifying MOMP had to be developed. The prohibitively small amounts of protein resulting from the growth of chlamydiae in cell culture also limited the methods of purification which

could be employed. Indeed, the techniques used for determination of structure and function were not compatible with several traditional purification protocols. These limitations prompted the adoption of many different, sometimes novel, techniques in the search for the most successful method of MOMP purification.

3.2 Purification of MOMP

3.2.1 SDS-PAGE and immunoblot analysis of OG-DTT-solubilised MOMP

SDS-PAGE and immunoblotting analyses of the MOMP-enriched fraction solubilised using OG-DTT illustrate the purity of the MOMP preparation used in planar lipid bilayer analysis (Chapter 4). Silver staining of this sample identified a 38 kDa protein corresponding to the MOMP, as well as contaminant proteins of approximately 90 kDa (Fig. 3.1). These contaminants were identified as the OMP90 (formerly POMP) family, which has been previously described (Longbottom *et al.,* 1998b), by Western blot analysis with OMP90-specific monoclonal antibodies (Fig. 3.2 [A]). Using the more sensitive Protogold protein stain, Western blots of the OG-DTT fraction revealed more clearly the contamination with OMP90 proteins, no other protein contaminants were visible (Fig. 3.2 [B]). Western blots of the OG-DTT solubilised MOMP-enriched fraction probed with mAbs specific for the oligomeric (A11 and 4/11, see Table 1) and monomeric (4/11) forms of MOMP demonstrated that under relatively non-denaturing conditions (MOMP is SDS-resistant if unboiled) MOMP is present as an oligomer with an apparent molecular mass of 100 kDa (Fig. 3.3), and that the MOMP-enriched fraction denatured by boiling gives rise to a 38 kDa monomer (Fig. 3.3). These results are in agreement with those of McCafferty *et al.* (1995).

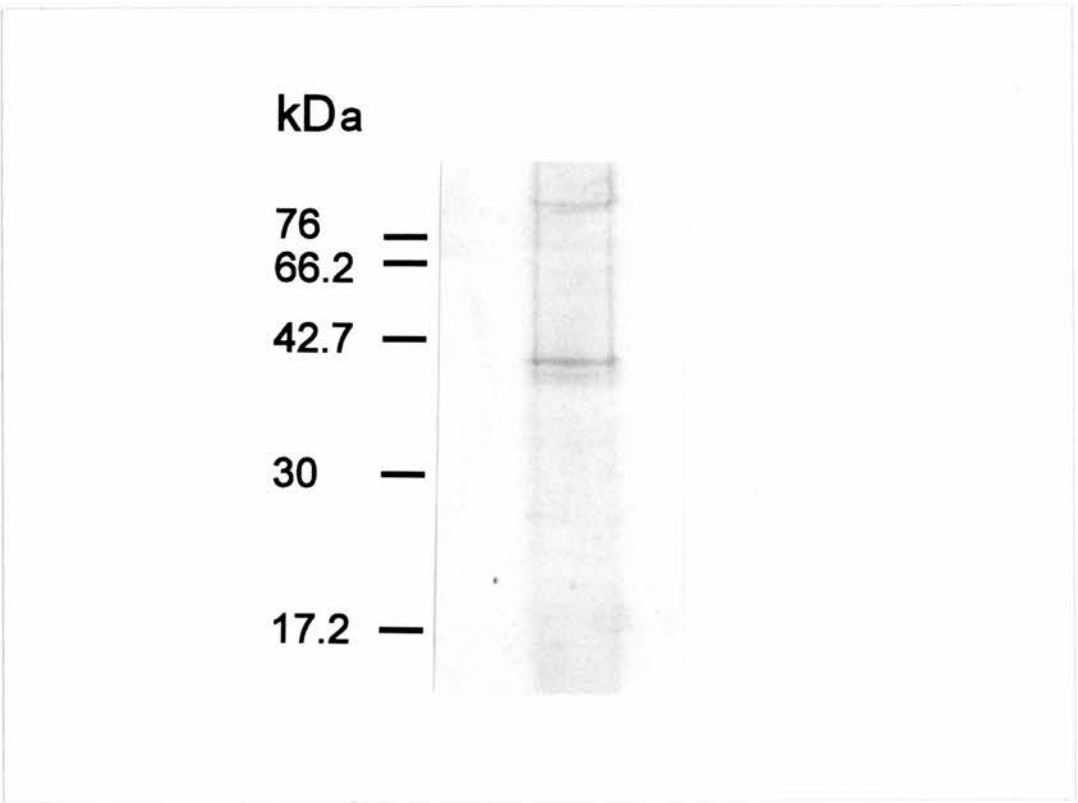
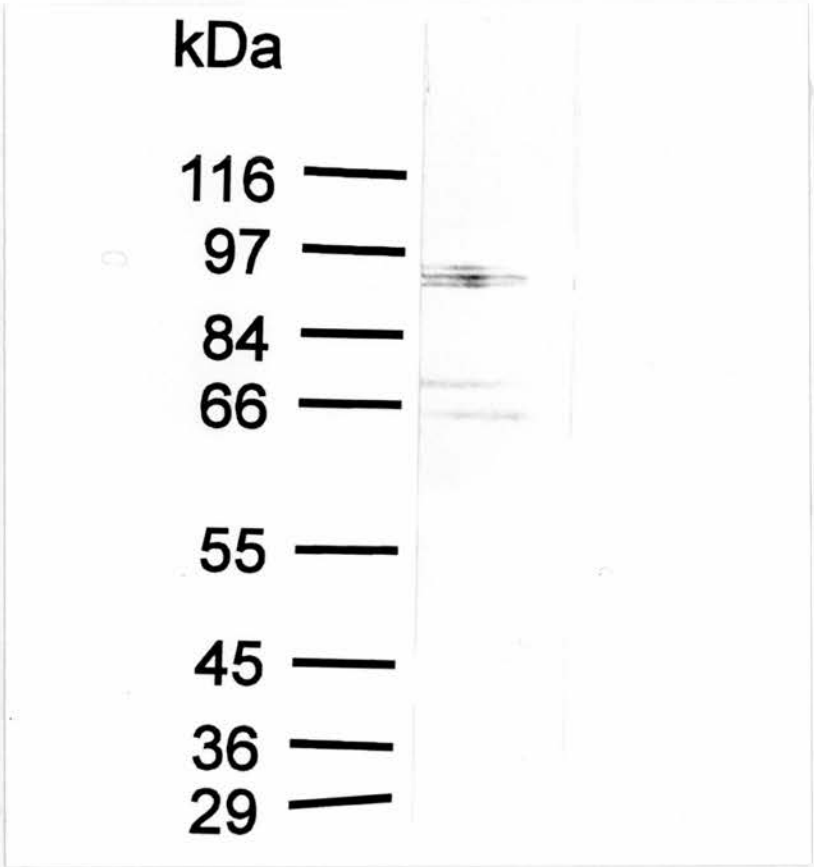


Figure 3.1 SDS-PAGE analysis of OG-DTT-solubilised MOMP-enriched preparations by silver staining. Molecular masses are indicated.

Figure 3.2 Western blot analysis of OG-DTT-solubilised MOMP-enriched preparations. The OG-DTT-solubilised MOMP-enriched preparation was subjected to SDS-PAGE on 12.5 % gels, immunoblotted, and probed with (**A**) mAbs 181 and 0040 and (**B**) total protein stain Protogold.

A



B

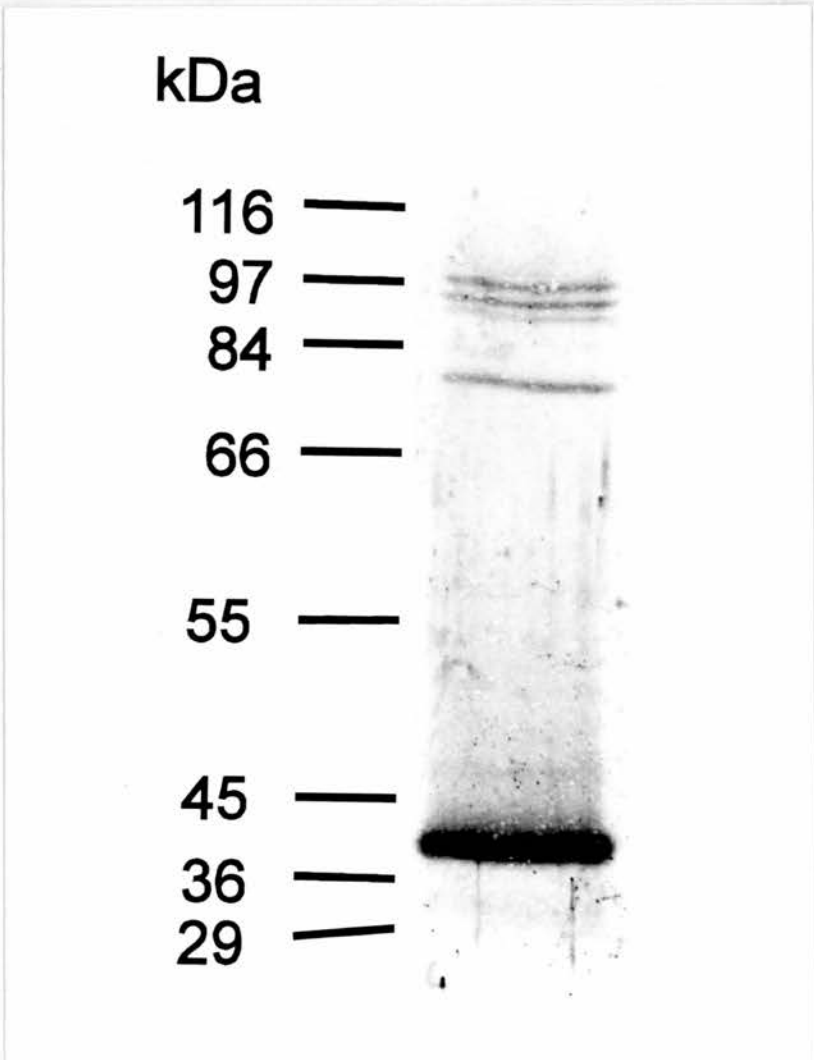
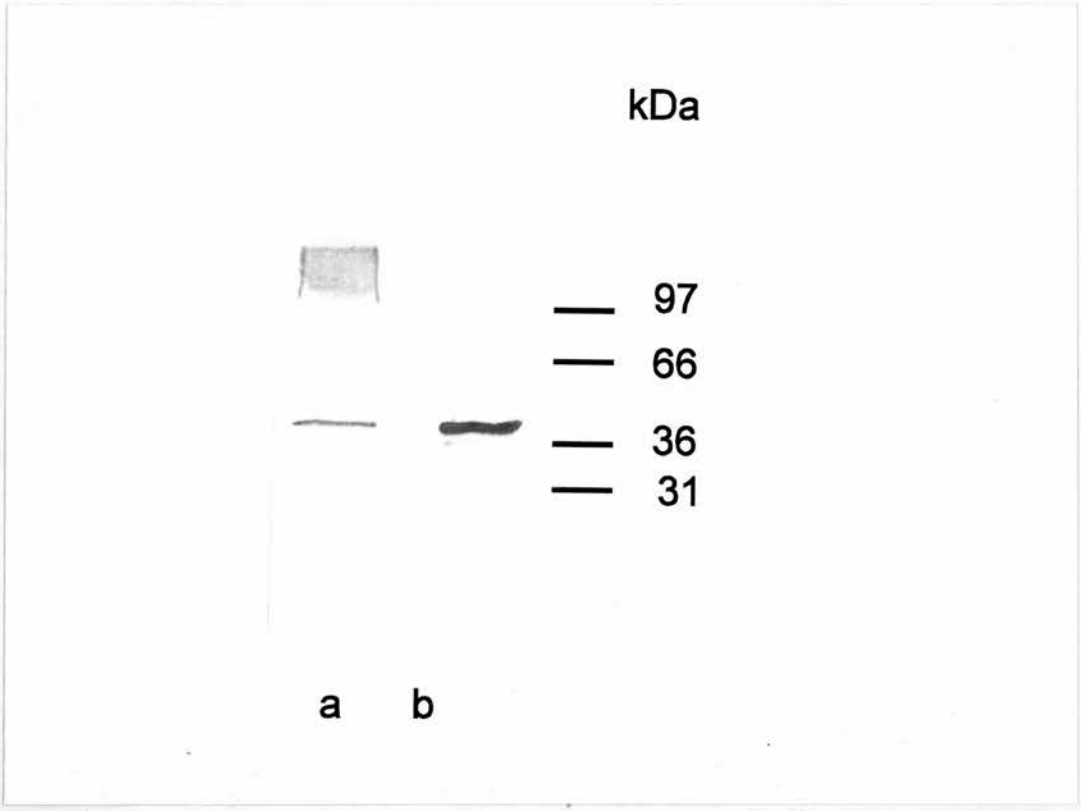
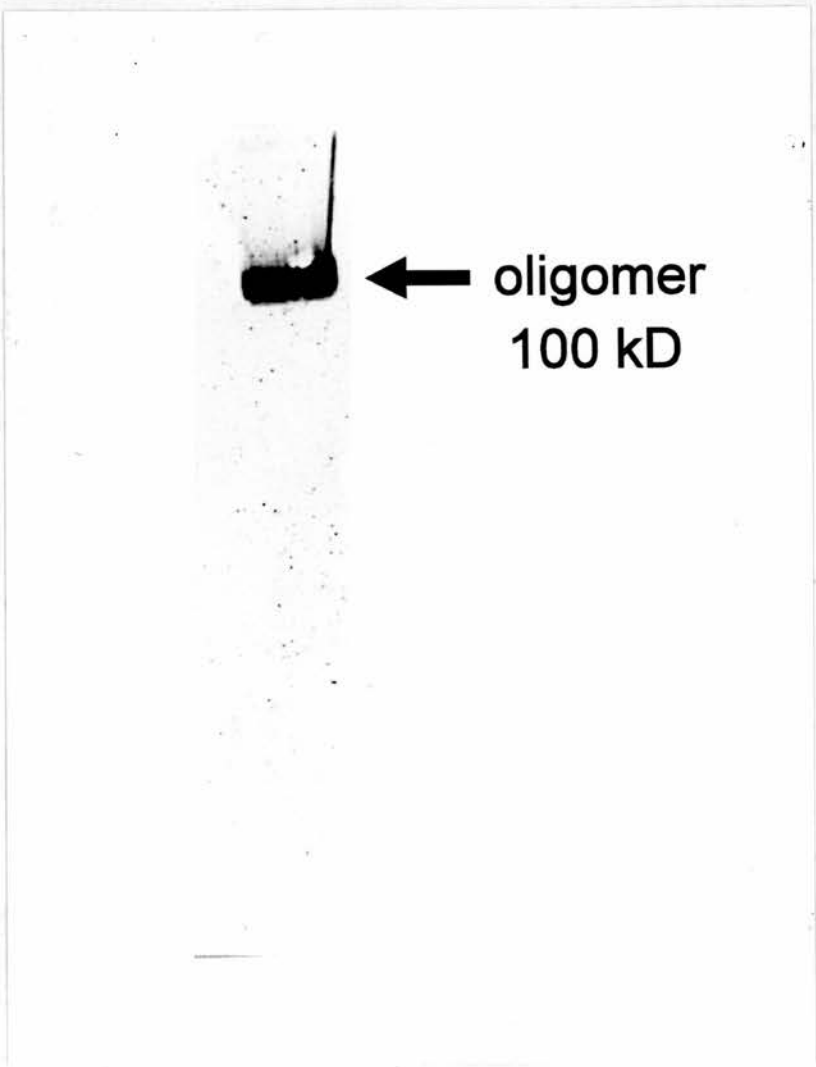


Figure 3.3 Western blot analysis of OG-DTT-solubilised MOMP-enriched preparations. The OG-DTT-solubilised preparation was subjected to SDS-PAGE on 12.5% gels and probed using (**A**) mAb 4/11 (sample loaded without boiling, lane (a) and sample loaded after boiling, lane (b)) and (**B**) mAb A11 (sample loaded without boiling). Molecular masses are indicated.

A



B



Western blot analysis of OG-DTT solubilised MOMP probed with monoclonal antibody 4/11 revealed an antigenic protein with a molecular weight of approximately 36 kDa (Figure 3.3 A [lane b]). Despite a very strong reaction when probed with 4/11, this protein was barely visible by Coomassie staining. N-terminal amino acid sequencing of this protein did not result in a decipherable sequence. It was hypothesised that this protein was the result of cleavage of the MOMP at a putative acid-labile site (Appendix I), however, incubation of recombinant tMOMP and electroeluted native MOMP in solutions with a pH as low as 2, to demonstrate acid lability, did not result in the appearance of a similar protein.

3.2.2 Differential solubilisation of MOMP

An attempt was made to solubilise MOMP from chlamydial outer membrane complexes using various detergents which it was hoped might improve the purity of recovered MOMP. SDS-PAGE analysis of fractions solubilised in 2% (w/v) solutions of the various detergents, as well as the resulting pellets, indicated that MOMP could only be solubilised from COMCs by incubation in SDS or OG. The presence of DTT was also found to be required for the solubilisation of MOMP. With all other detergents MOMP was found in the pellet. Indeed, OG was only able to solubilise MOMP at a critical concentration of 2% (w/v), at lower concentrations MOMP was recovered in the pellet. As estimated by densitometry, the SDS solubilised MOMP accounted for only 65% of the total protein content, while OG-solubilised MOMP accounted for 80%. These detergent solubilised fractions were found to contain many other contaminant proteins in addition to MOMP, as observed by Coomassie staining.

3.2.3 Isoelectric focusing

Isoelectric focusing on precast polyacrylamide, non-denaturing IEF gels (pH 5-7) resulted in the focusing of OG-DTT solubilised MOMP between pH 5-6, as estimated using standard pI markers, and visualised by silver staining (Fig. 3.4). In addition, analysis of this OG-DTT solubilised MOMP by 2-D gel electrophoresis (pH3-10) resulted in the focusing of MOMP at pH 5.5, again estimated using standard markers. The OMP90 proteins, also present in the OG-DTT solubilised sample, were not visably focused by either of these methods.

Preparative isoeloectric focusing, using the MinipHor protein fractionator, was attempted to further purify MOMP from the OG-DTT solubilised fraction, on the basis of pI. SDS-PAGE and Western blotting analyses of the fractions eluted from the 0.8% pH 4-7 and 0.2% pH 3-10 gradient revealed that MOMP tightly focused in fractions ranging from pH 5-6. The 90 kDa proteins appeared focused over several fractions ranging from pH 4-7. Silver staining and Western blots probed with mAbs 4/11 or 0040 and 181 (anti-OMP90 proteins, see Table 1) were used to visualise eluted protein. However, recovery of focused MOMP was only 45% and so this protocol was not used routinely for purification.

3.2.4 Electroelution

SDS-PAGE and immunoblot analysis of the MOMP-enriched fraction solubilised using OG-DTT under relatively non-denaturing conditions (Section 2.2), revealed a 100 kDa band which reacted with anti-MOMP mAb 4/11. This band was successfully electroeluted, resulting in an eluted MOMP concentration of approximately 0.03 mg/ml. The recovery rate was estimated at 60 %. Using the more sensitive Protogold protein stain, Western blots of the electroeluted MOMP

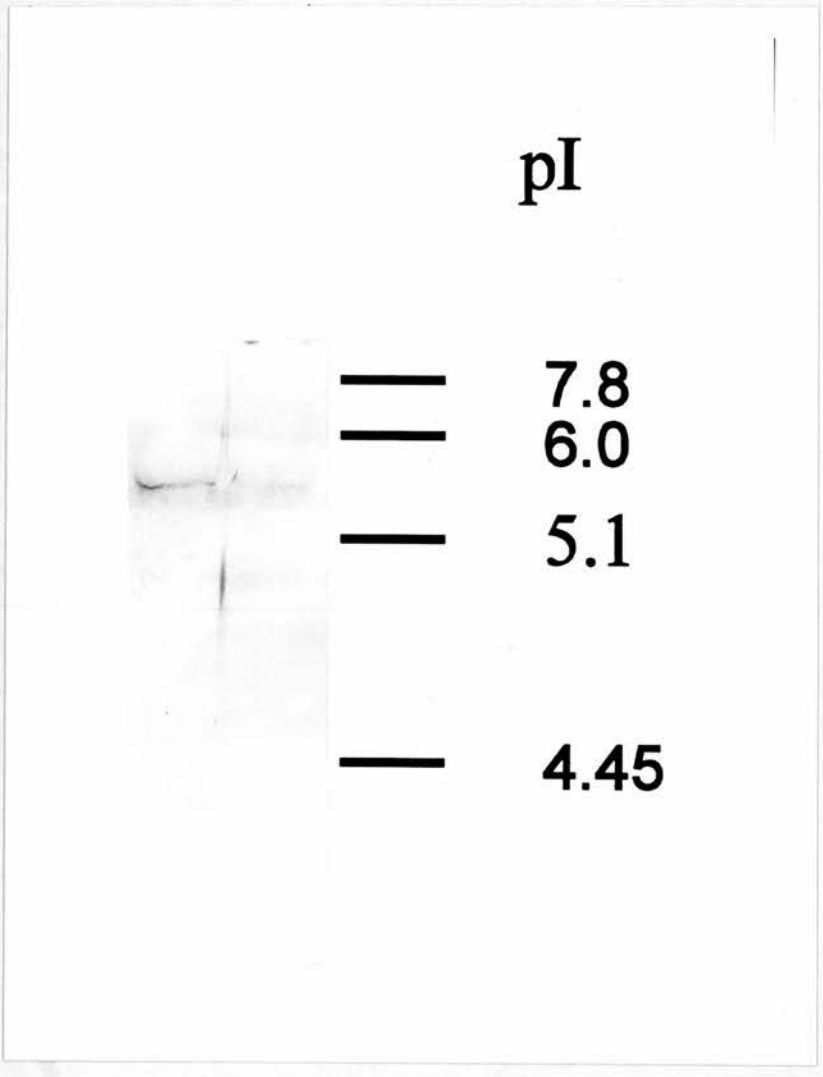


Figure 3.4 Isoelectric focusing of OG-DTT-solubilised MOMP-enriched preparations. OG-DTT-solubilised MOMP was focused on a pre-cast 5% (w/v) polyacrylamide, non-denaturing gel with an ampholyte range of pH 3-10and silver stained. pI markers are indicated.

fraction revealed no other protein contaminants. Electroeluted MOMP was used in planar lipid bilayer studies (Chapter 4), however it could not be routinely reconstituted in bilayers due to the presence of SDS in the sample.

3.2.5 Sucrose density gradient centrifugation

The pellet and 1 ml fractions resulting from the centrifugation of 0.1 ml OG/DTT solubilised MOMP through a 5-20% (w/v) linear gradient of sucrose (Section 2.4.2) were solubilised in Laemmli buffer and analysed by SDS-PAGE (Fig. 3.5). Silver staining revealed that the pellet contained at least 80% MOMP, as estimated by densitometry. MOMP was not present in the fractions of the sucrose gradient. It was impossible to solubilise this MOMP-rich pellet by any other method than boiling in Laemmli buffer. In an attempt to stop the pelleting of MOMP at the base of the sucrose gradient, OG-DTT solubilised MOMP was centrifuged into a 60% (w/v) Nycodenz cushion. However, MOMP was again pelleted through this cushion. The pelleting of MOMP through a sucrose gradient, and indeed through a 60% (w/v) Nycodenz cushion, illustrates its propensity to form high molecular weight oligomers. Unfortunately, our inability to solubilise the MOMP-enriched pellet, without boiling in SDS sample buffer, meant that this relatively pure sample could not be used for structural analysis or planar lipid bilayer reconstitution studies.

3.2.6 Hydroxyapatite chromatography

The most successful method for purifying native, conformational MOMP was hydroxyapatite chromatography in the presence of SDS. Figure 3.6 shows the silver stained SDS-PAGE analysis of fractions eluted from a hydroyapatite column

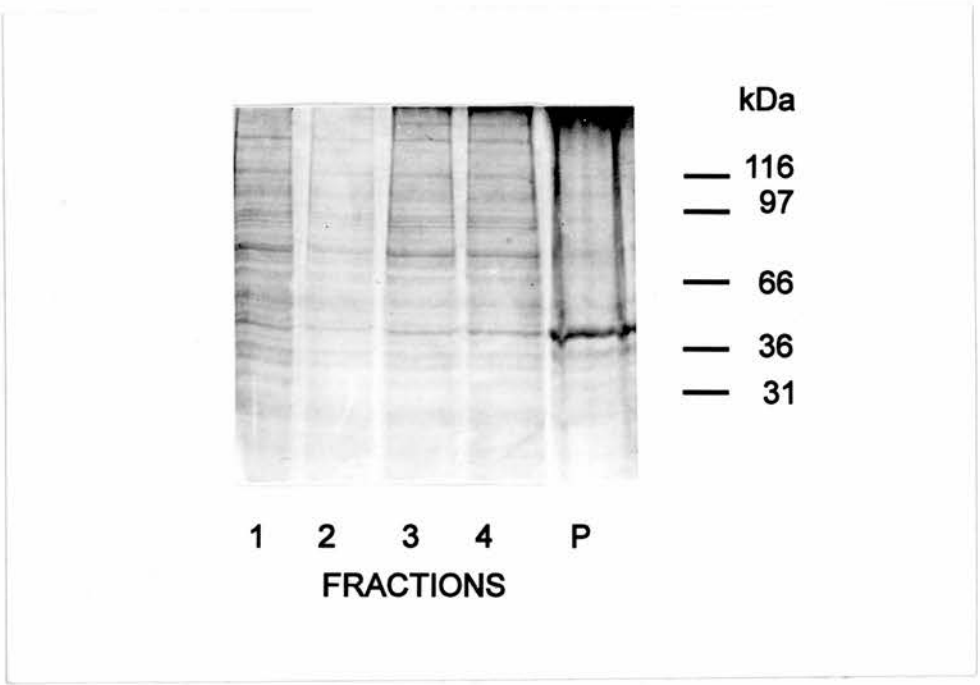


Figure 3.5 SDS-PAGE analysis of fractions and pellet resulting from centrifugation of OG-DTT-solubilised MOMP through a sucrose density gradient. OG-DTT-solubilised MOMP was loaded onto a 5-20% (w/v) sucrose gradient and centrifuged at 130,000 x g for 16 h. The resulting pellet and fractions were subjected to SDS-PAGE analysis and silver stained (samples loaded without boiling). Molecular masses are indicated.

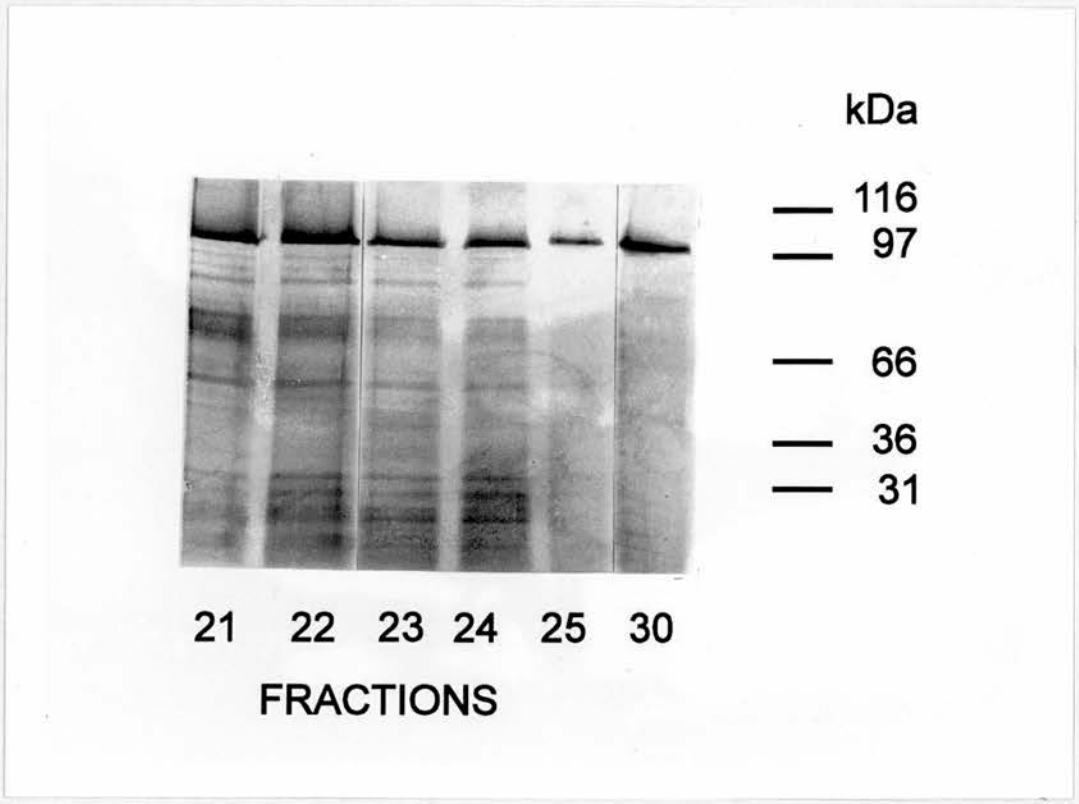


Figure 3.6 SDS-PAGE analysis of the hydroxyapatite-purified MOMP-enriched preparation. Fractions eluted from the hydroxyapatite column (5 by 1 cm) loaded with the 2% (w/v) SDS-solubilised MOMP-enriched preparation were subjected to SDS-PAGE on a 12.5% gel, under reducing conditions.

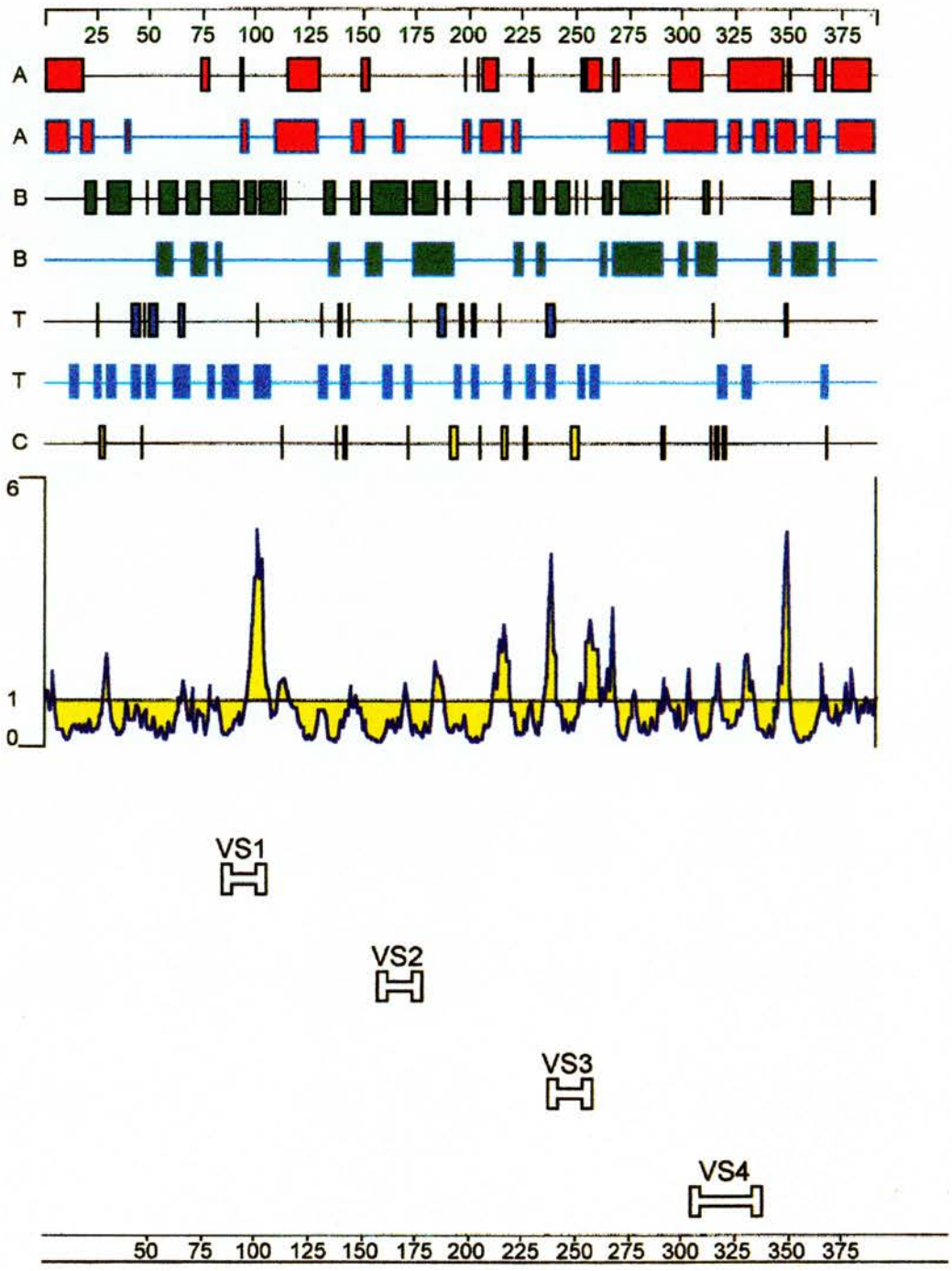
under relatively 'non-denaturing' conditions (Section 2.4.5). Although fractions 20-30 contained significant amounts of MOMP oligomer (100 kDa) only fractions 25-30 contained very few contaminant proteins. MOMP oligomer accounted for >90% of the hydroxyapatite purified protein therefore enabling structural analysis by CD to be carried out (Section 3.3.2). Unfortunately, the protocol used to purify MOMP for structural studies could not be employed during the purification of MOMP for planar lipid bilayer analysis due to the destabilising effects of SDS on the bilayer. Dialysis to remove SDS from hydroxyapatite purified fractions was not feasible because of the small amounts of protein involved.

3.3 Structural analysis and characterisation of MOMP

3.3.1 Secondary structure prediction

Protein sequence analysis of MOMP from the OEA isolate of *C. psittaci* using DNASTAR sequence analysis software prediction methods (Section 2.6.1) produced conflicting results (Fig. 3.7). Garnier-Robson analysis (Garnier *et al.*, 1978) predicted the presence of substantial β-structure (42% β-sheet, 33% α-helix, 13% random coil and 12% β-turn). The Chou-Fasman alogarithm, based on the 64 protein method (Chou and Fasman, 1978) and predicting 80% accuracy in assigning secondary structure, estimated that the structure of MOMP was predominantly α-helix (56% α-helix, 30% β-sheet and 14% β-turn). Significantly both these prediction alogarithms suggest the presence of large amounts of β-sheet structure within MOMP. However, the inaccuracy of such methods in predicting β-structure is well documented.

Figure 3.7 Secondary structure predictions of the OEA *C. psittaci* MOMP protein sequence. Variable segments of the sequence are represented (VS).


25 50 75 100 125 150 175 200 225 250 275 300 325 350 375
A
A
B
B
T
T
C
6
1
0
VS1
VS2
VS3
VS4
50 75 100 125 150 175 200 225 250 275 300 325 350 375
Alpha, Regions - Garnier-Robson
Alpha, Regions - Chou-Fasman
Beta, Regions - Garnier-Robson
Beta, Regions - Chou-Fasman
Turn, Regions - Garnier-Robson
Turn, Regions - Chou-Fasman
Coil, Regions - Garnier-Robson
Surface Probability Plot - Emini

3.3.2 Secondary structure determination

Oligomeric MOMP, purified to >90% by hydroxyapatite chromatography (Section 3.2.6), in the presence of SDS, achieved the criteria for accurate structural analysis by CD. Fractions 25-30, which were eluted at a concentration of approximately 0.3M sodium phosphate, were subjected to CD analysis in the far-UV region (Price, 1996). The CD analysis of fraction 25, determined to have a protein concentration of 0.053 mg/ml, as estimated by densitometry, is shown in Figure 3.8. The spectrum of purified protein is consistent with the presence of a large percentage of β-structure, and prediction using the CONTIN procedure (Provencher and Glockner, 1981) estimated 62 % +/- 10% β-structure, 38% +/- 10% random coil and α-helix at 0% +/- 1.8%.

CD in the far-UV region was also performed on a sample of OG-DTT solubilised MOMP (results not shown), estimated to have a protein concentration of 0.1 mg/ml. MOMP comprised approximately 80% of the total protein content. Secondary structure estimations, again calculated using the CONTIN procedure, were: α-helix 47% +/-2.1%, β sheet 34% +/-2.5% and random coil 19% +/- 4.1%.

3.3.3 Association of LPS and MOMP oligomers

The close association between MOMP and LPS, demonstrated by cross-linking studies (Birkelund *et al.,* 1988), prompted us to look for the potential association of LPS with the oligomeric form of MOMP. In the case of many bacterial porins, oligomeric structure is thought to be stabilised, or even maintained, by a close association with a LPS moiety. This may also be the case with MOMP. The results of immuno-blotting analyses of OG-DTT-solubilised MOMP,

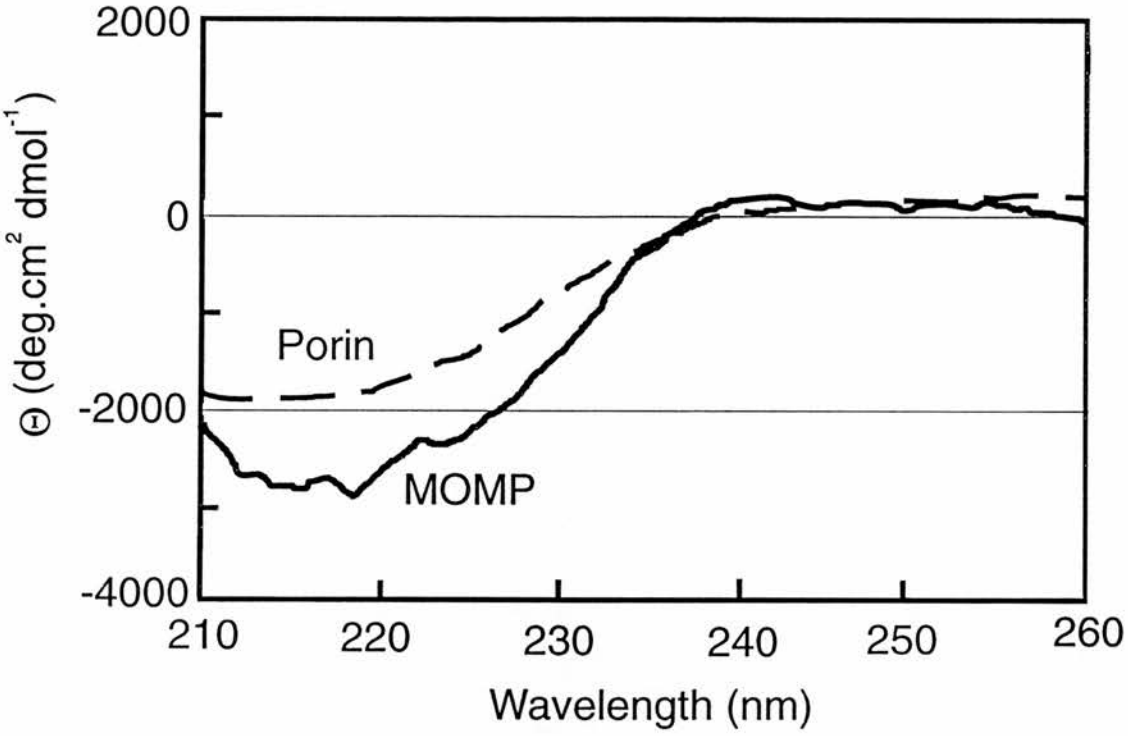


Figure 3.8 Circular dichroism spectra of hydroxyapatite-purified MOMP and bovine heart mitochondrial porin in the presence of 0.1% (w/v) OG.

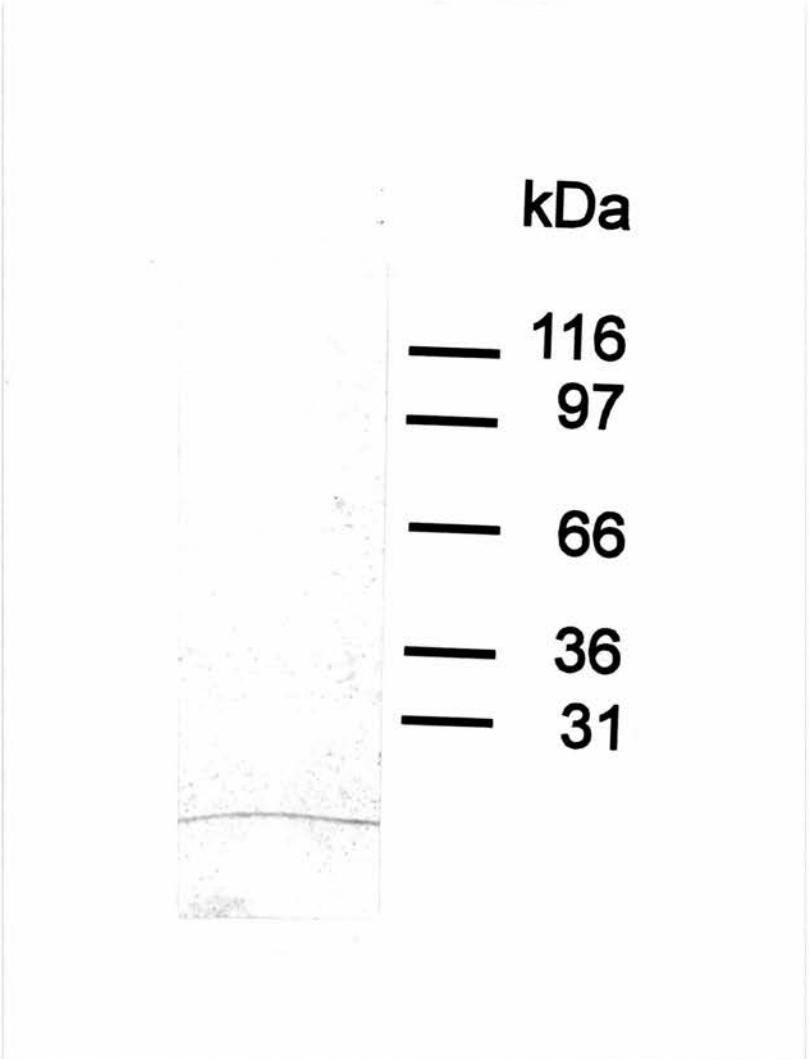


Figure 3.9 Western blot analysis of the OG-DTT-solubilised MOMP-enriched preparation. The OG-DTT-solubilised MOMP-enriched preparation was subjected to SDS-PAGE on 12.5% gels, immunoblotted, and probed with mAb 13/4 (sample loaded without boiling).

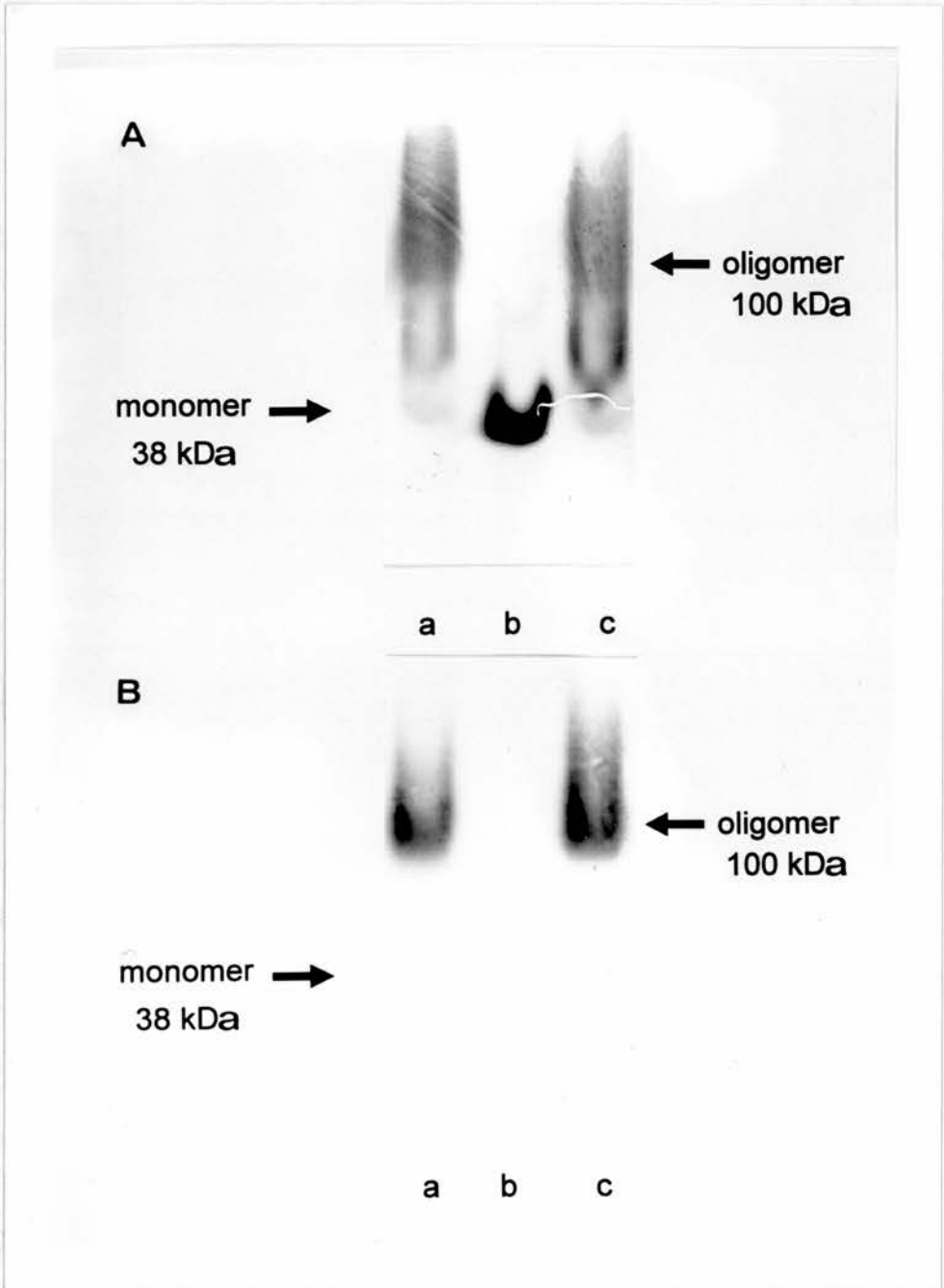


Figure 3.10 Western blotting transfer properties of oligomeric and monomeric MOMP. OG-DTT-solubilised MOMP was subjected to SDS-PAGE on 12.5% gels, transferred similtaneously onto two PVDF membranes (**A** and **B**), and probed with mAb 4/11 (samples a and c loaded without boiling, sample b loaded after boiling

transferred onto PVDF membrane under relatively non-denaturing conditions and probed with an LPS-specific monoclonal antibody, 13/4, are shown in Figure 3.9. No LPS was found to associate with oligomeric MOMP at 100 kDa, instead it migrated in the dye front. Therefore, close association of LPS would not appear to be essential in the maintenance of oligomeric assemblies of MOMP. However, Western blot analysis of MOMP under non-denaturing conditions and probed with MOMP-specific monoclonal antibody 4/11 (Fig. 3.3 A [lane b]) revealed the continued presence of a small, but significant quantity of monomeric MOMP (38 kDa) in addition to oligomeric MOMP. It is conceivable that LPS migrating in the dye front originates from these dissociated MOMP oligomers.

3.3.4 Western blotting transfer properties of oligomeric MOMP

An interesting observation was made during the transfer of non-denatured and denatured MOMP onto PVDF membranes by overnight wet blotting (Section 2.3.2). Two membranes were placed in the blotting sandwich and the transfer of both monomer and oligomer onto these membranes was monitored by probing with MOMP-specific mAb, 4/11 (Fig. 3.10). Interestingly, the 100 kDa oligomer was observed to transfer straight through the first membrane onto the second upon immunoblotting. In contrast, monomeric MOMP was only detectable on the first membrane.

3.4 Discussion

As discussed previously (Section 3.1), the purification of MOMP for structural and functional analyses is limited by the requirement to maintain native structure and by the inability to refold this highly disulphide bond crosslinked,

hydrophobic protein to native conformation. Perhaps the most common method of obtaining enriched preparations of native MOMP is via solubilisation of chlamydial outer membrane complexes using OG-DTT (Bavoil *et al.,* 1984). These highly enriched MOMP preparations are, however, contaminated by the OMP90 family of proteins which have recently been localised to the chlamydial outer membrane (Longbottom *et al.,* 1998a). The yield of MOMP from this protocol can often be poor, with large quantities of MOMP being lost in the sarkosyl/DTT solubilisation step. This may be attributed to a high ratio of RBs/EBs in the tissue culture harvest. MOMP is not highly disulphide bond cross-linked in the RB developmental form and sarkosyl/DTT may be sufficient to solubilise the majority of this MOMP, in addition to a vast number of other membrane associated proteins. SDS-PAGE and Western blotting analyses of the OG-DTT-solubilised sample confirm that under relatively 'non-denaturing' conditions, MOMP forms a 100 kDa oligomer. However, the determination of molecular weight under these conditions is unreliable. Indeed, the term SDS-resistant, applied to proteins apparently forming high molecular mass oligomers in the absence of boiling in SDS sample buffer, is misleading. It may be the case that such proteins are not 'fully' denatured in the presence of SDS and the resulting mis-folded proteins appear as high molecular weight oligomers upon SDS-PAGE. However, in the case of MOMP, there is supplimentary evidence suggesting that it spontaneously forms oligomeric structures, most probably trimers. The results of sucrose density gradient centrifugation by McCafferty *et al.* (1995) determined that MOMP had a sedimentation coefficient equivalent to that of porin proteins which have had their trimeric structure confirmed by x-ray crystallography (Cowan, *et al.,* 1992; Schulz, 1996).

The propensity of MOMP to form high molecular weight oligomers was further illustrated by the pelleting of OG-DTT solubilised MOMP following centrifugation through a 5-20% (w/v) sucrose density gradient. This technique was used as an attempted method of MOMP purification and was successful in that the recovered pellet contained >90% MOMP. However, the pellet could not be re-solubilised in a form that would maintain structural information and therefore could not be used in the pursuit of the two main aims of structural analysis by CD and planar lipid bilayer reconstitution.

The Western blotting transfer properties of the oligomeric and monomeric forms of MOMP are markedly different. The high molecular weight oligomer transfers through PVDF membrane much more rapidly than the 38 kDa monomer. This observation may be explained by the increased number of exposed hydrophobic regions in the denatured protein in comparison to the more folded oligomer.

Immunoblotting of OG-DTT solubilised MOMP with the MOMP-specific mAb 4/11 revealed strong reactivity with a 36 kDa protein. Analysis of the MOMP primary sequence revealed the presence of a putative N-terminal acid-labile site. Mild acidic conditions are known to cause hydrolysis of polypeptides at sites distal to Asp-Pro residues (Landon, 1977) and it was hypothesised that this highly immunogenic protein resulted from such a reaction in MOMP. Should this have been the case, it would have posed interesting questions as to the possibility of this cleavage reaction taking place in the mildly acidic environment of the inclusion body. Would this Asp-Pro site be sufficiently exposed in the folded protein to allow the cleavage reaction to take place? If so, what would be the effect of such a reaction on the conformation of MOMP and what would be the physiological

implications? In an attempt to begin answering some of these questions the 36 kDa protein was N-terminally sequenced to ascertain if the protein was, indeed, cleaved at the Asp-Pro site. Unfortunately, no decipherable sequence could be obtained. In addition, incubation of recombinant MOMP in acidic conditions did not result in cleavage suggesting that the highly immunogenic protein does not result from acid hydrolysis. Alternatively, it may be the case that the 36 kDa protein results from the aberrant folding of MOMP which has not been entirely denatured by boiling in SDS sample buffer. Mis-folding may have further exposed the site recognised by 4/11 explaining the strong reaction of this mAb upon immunoblotting.

CD analysis of hydroxyapatite-purified MOMP confirmed the presence of a large proportion of β-structure (62%) (Provencher and Glockner, 1981), as suggested by secondary structure prediction alogarithms (Section 3.3.1). This level of β-structure is very similar to that observed in gram negative bacterial porin proteins. Several porins have been crystallised and subjected to x-ray diffraction leading to the development of a generalised porin structural model which may be applicable to MOMP (Cowan *et al.,* 1992). Each porin subunit is thought to consist of 16 transmembraneous β strands with lengths ranging from 6 to 17 residues. The 16 strands form a completely anti-parallel β barrel, in which all strands are connected to their neighbours. These porin subunits associate to form a trimer of β barrels giving rise to three distinct diffusion channels through the outer membrane. Whether or not this model can be applied to MOMP remains to be seen. The only previously described model depicting a possible structural arrangement of MOMP in the outer membrane was proposed by Baehr *et al.* (1988). Using information from immuno-mapping and proteolytic digestion studies, they suggested that

MOMP is arranged as 8 anti-parallel transmembrane spanning regions across the membrane with the 4 VS forming surface exposed loops. Clearly this arrangement of MOMP is unfeasible. In this model the predicted transmembrane spanning regions range from approximately 28 to 51 residues in length. Even if a transmembrane β-sheet was angled in relation to the plane of the membrane, it would be extremely unlikely to reach such lengths. Indeed, the length of these proposed membrane spanning regions would also exceed the number of residues required to form a transmembrane α-helix (20-25 residues). The generalised 16 strand anti-parallel β-sheet model of the porin proteins has been mapped onto the MOMP sequence and is discussed in greater detail in Chapter 7. The relatively high proportion of random coil, calculated from the MOMP spectrum, may be attributed largely to its four surface exposed variable segments (Stephens *et al.,* 1986).

CHAPTER FOUR

RESULTS:

PLANAR LIPID BILAYER RECONSTITUTION OF NATIVE MOMP FROM THE OEA SUBTYPE OF *CHLAMYDIA PSITTACI*

4.1 Introduction

The 40 kDa MOMP is a common component of all 4 species of *Chlamydia* and is present in both the EB and RB developmental forms. MOMP has been studied in great detail since the landmark discovery that the protein, recovered from SDS gels, was capable of raising antibodies which could neutralise the infectivity of *C. trachomatis in vitro* (Caldwell and Perry, 1982). Vaccine preparations based on chlamydial outer membrane complexes, highly enriched for MOMP in its native form, have been shown to be protective against chlamydial disease in sheep (Tan *et al.,* 1990), guinea pigs (Batteiger *et al.,* 1993) and mice (de Sa *et al.,* 1995; Pal *et al.,* 1997). Most recently, protection in mice was demonstrated using a DNA vaccine comprising only the MOMP gene (Zhang *et al.,* 1997). Clearly, these results make MOMP the primary candidate for a sub-unit vaccine against chlamydial infection.

Despite the understandable interest in MOMP, resulting in many years of intensive study, there has yet to be a definitive functional assignment to this protein. As discussed in Section 3.1, problems purifying native chlamydial MOMP or obtaining recombinant MOMP in a native form have hampered the study of a structure/function studies. However, MOMP has been implicated in many aspects of chlamydial pathogenesis and has been suggested to be multifunctional. Its predominance in the outer membrane of *Chlamydia* (>60% of the total protein mass), has resulted in the investigation of MOMP's role in the infectious process (Su *et al.,* 1996). In addition, MOMP is believed to play a significant part in maintaining the structural integrity of the outer membrane of the EB (Newhall and Jones, 1983). The cysteine rich proteins of the chlamydial envelope, including MOMP, are believed to form a disulphide bond crosslinked supramolecular

structure, which may act as a functional replacement for the traditional peptidoglycan S-layer (Hatch, 1996).

Bavoil *et al.*(1984) used the liposome swelling technique to demonstrate that the chlamydial outer membrane contained pores and, due to its predominance in the outer membrane, MOMP was proposed as the likely pore-forming protein. With subsequent cloning and sequencing of the MOMP gene (Stephens *et al.,* 1986), it became clear that MOMP has an acidic isoelectric point and charge distribution appropriate to a porin protein. Sequence comparisons of MOMP from different serovars combined with immunochemical analysis have identified variable surface exposed segments of the molecule similar to those found in other porins. More recently, MOMP solubilised in OG-DTT was shown to form an oligomer, with electrophoretic and sedimentation properties consistent with a trimeric structure (McCafferty *et al.,* 1995). These oligomers resisted complete denaturation with SDS in a way similar to classical gram-negative porin molecules, which are also trimers. The primary aim of this study was to carry out a direct test of the 'porin channel' hypothesis at the molecular level by reconstitution of native, oligomeric MOMP into the planar lipid bilayer system.

4.2 Planar lipid bilayer analysis of MOMP

4.2.1 Planar lipid bilayer reconstitution

OG-DTT solubilised MOMP (Fig. 3.1) incorporated spontaneously into planar lipid bilayers within 5-10 min of addition, to give rise to ion channel-like unit currents. The process of insertion was unaffected by the presence of a positive or negative holding potential. Bilayers were cast primarily from a decane suspension of diphytanoyl phosphatidylcholine (chosen because it is a very robust bilayer lipid),

however, MOMP bilayer incorporation was unchanged when alternative lipids were used. The resulting unit currents were not seen at 0 mV, despite the presence of an ion gradient (250 mM KCl *versus* 50 mM KCl, see Section 2.10.1). This suggested that channel opening was a voltage-dependent process. Channel incorporation into the bilayer appeared to be autocatalytic, in that the rate of incorporation accelerated markedly following the initial appearance of unit currents. After the first evidence of channel incorporation, the solution in the *cis* chamber was changed by perfusion to prevent further MOMP bilayer fusion, effectively limiting the channel content of the bilayer. Finally, it was confirmed that the addition of equivalent amounts of OG-DTT alone had no effect.

Figure 4.1 shows bilayer traces, with the same solutions on both the *cis* and *trans* side of a bilayer that had been exposed to OG-DTT solubilised MOMP. The data were obtained by switching the holding potential from 0 mV to either +80 mV or -80 mV as indicated. Bursts of channel openings are superimposed on bilayer capacitative transients (Section 2.10.1). These bursts of opening are typical, with unresolvably-fast activation of multiple unit conductances. Following this burst of activation, the maintainence of high holding potentials resulted in the voltage-dependent closure of the activated channels. This inactivation process was repeatedly shown to be asymmetric between positive *versus* negative holding potentials, suggesting the unit channels had a common functional (and therefore, presumably, a defined structural) orientation. Normally, the appearances on switching were similar to those shown in Figure 4.1, with more open/closed transitions at negative compared to positive potentials, but in some experiments all the channels became incorporated in the "opposite" orientation. In addition, the

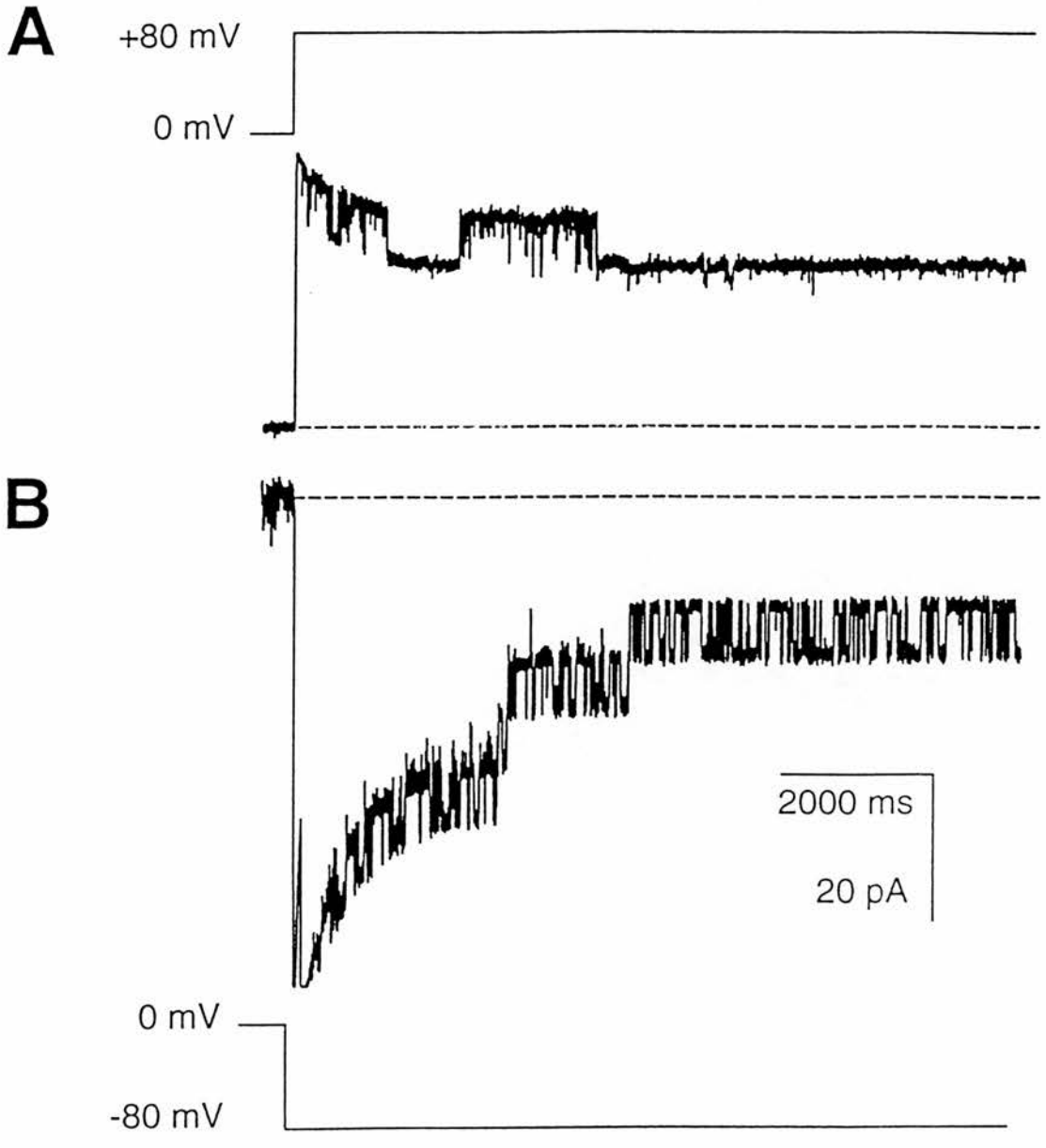


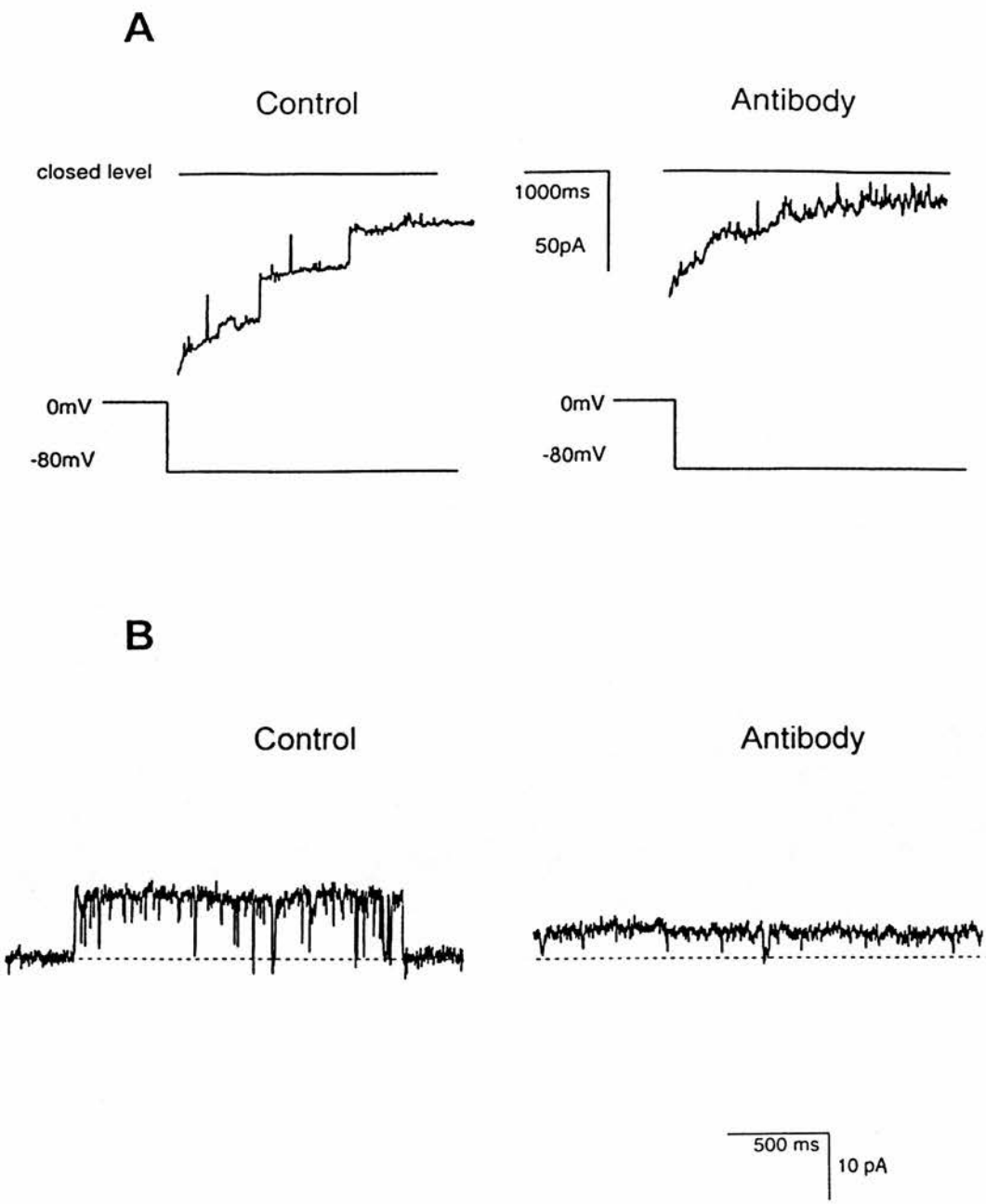
Figure 4.1 Ion channel recordings from OG-DTT-solubilised MOMP in a bilayer bathed in 50 mM KCl-10 mM Tris-HCl (pH7.4). (**A**) Holding potential switched from 0 to +80 mV. (**B**) Holding potential switched from 0 to -80 mV. Recordings were low-pass filtered at 100 Hz and show decaying capacitative transients representing the discharge of the typically large bilayer capacitance transient (~300 pF).

bursts of activity seen after switching to negative holding potentials normally contained more unit currents than those seen at positive potentials. Channel openings elicited by switching to positive holding potentials declined to a "steady-state" equilibrium level of activity more rapidly than bursts elicited by switching to negative holding potentials. The use of a robust bilayer lipid enabled membrane potentials to be clamped as high as +/- 200 mV without breakage, however, the maximum number of unit currents generally appeared to occur at +/- 60 mV or above.

4.2.2 Identification of MOMP as the channel forming protein

Two types of experiment confirmed that MOMP was the channel-forming protein within the OG-DTT solubilised sample. Firstly, identical channel behaviour (and an identical rate of channel incorporation) was obtained using similar quantities of MOMP that had been highly-purified, either by hydroxyapatite chromatography (Section 3.2.6), or by excision and electroelution from SDS-PAGE gels (Section 3.2.4), as seen with OG-DTT solubilised MOMP. The hydroxyapatite purified sample (Fig. 3.6) was known to contain >90% MOMP, while MOMP constituted 100% of the total protein content of the electroeluted sample (both estimated by densitometry). Reconstitution of these MOMP samples into the bilayer resulted in the appearance of unit channels identical in amplitude and behaviour to those observed upon incorporation of the OG-DTT solubilised sample. Switching holding potentials from 0 mV to +/- 80 mV caused the same unresolvably fast activation of multiple unit conductances followed by successive, voltage-dependent channel closure. Unfortunately, these highly purified samples of MOMP could not be reconstituted into the bilayer routinely, as both contained SDS, which

Figure 4.2 Effects of mAb A11 on the MOMP channel. The bilayer was bathed in 50 mM KCl-10 mM Tris-HCl (pH7.4): mAb A11 was added to both the *cis* and *trans* chambers to a final dilution of 1/1000 in the presence of BSA (1mg/ml). (**A**) Traces obtained after switching the holding potential from 0 to -80 mV before (left) and 30 min after (right) the addition of mAb A11. In each record, there is a typical bilayer capacitative current transient superimposed on channel openings. (**B**) Detailed appearance of an individual unit current before (left) and after (right) the addition of A11. The dotted line represents the baseline level, corresponding to the closure of the unit conductance. Currents were filtered at 100 Hz.


A
Control
Antibody
closed level
1000ms
50pA
0mV
-80mV
0mV
-80mV
B
Control
Antibody
500 ms
10 pA

destabilises the bilayer. It was not possible to remove SDS from these fractions by dialysis due to the prohibitively small amounts of protein involved.

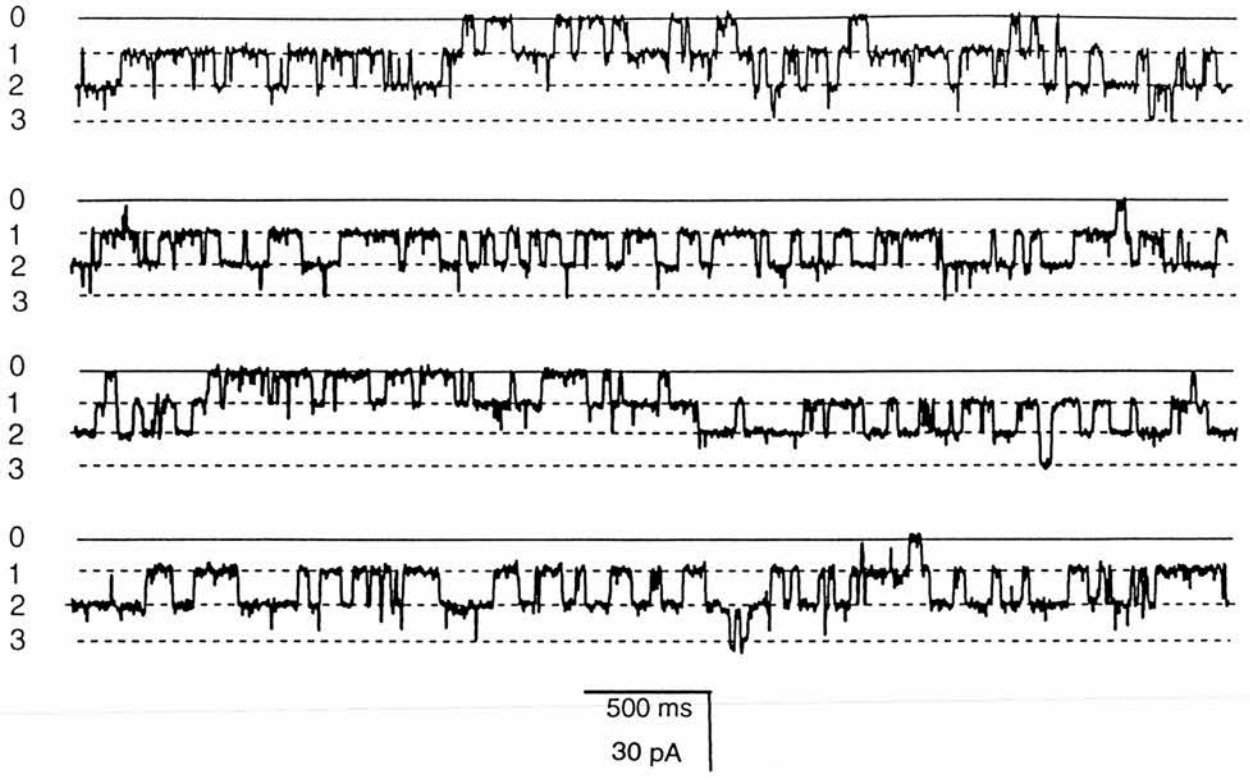
Secondly, addition of a MOMP-specific monoclonal antibody, A11 (Table 1), affected both the open/closed transitions of the channel and the amplitude of the unit currents (Fig. 4.2). Note that channels did not close completely in the presence of A11. These effects could not be reversed by perfusion with antibody-free solutions over periods up to 30 min. Monoclonal antibody A11 is oligomer specific in that it recognises only the oligomeric form of MOMP, not the denatured monomer, by Western blotting analyses. Not only do these results confirm that MOMP is the pore-forming protein of chlamydial outer membranes, they also suggest that MOMP is arranged as an oligomer within the bilayer, consistent with the proposed arrangement of MOMP in the outer membrane. Interestingly, the addition of antibody 4/11 to bilayers containing OG-DTT solubilised MOMP had no observable effect on the behaviour or amplitude of the resulting unit conductances. MAbs 181 and 0040 (Table 1), specific to the *C. psittaci* OMP90 family of proteins which are the main contaminants within the OG-DTT solubilised sample (Fig. 3.2), had no observable effect on the characteristics of the unit currents. Additions of other mAbs or 1 mg/ml BSA were also without effect (results not shown).

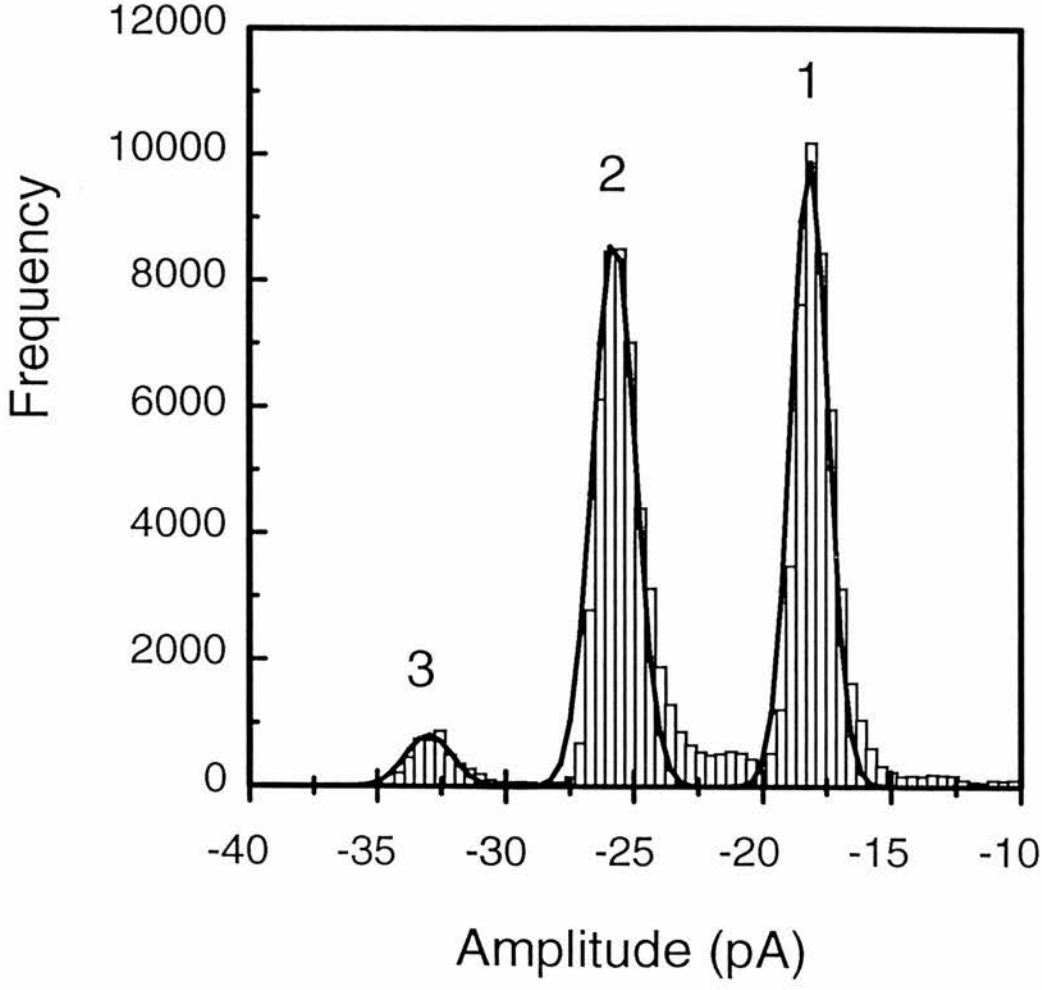
4.2.3 Properties of unit MOMP channels

Given that native MOMP tends to migrate as an oligomer, presumably a trimer, under relatively non-denaturing conditions on SDS-PAGE, and knowing that many classical porins are trimers, we measured the number of functional unit currents that could be obtained following bilayer reconstitution of OG-DTT solubilised MOMP. These individual conducting units of the channel (eg. levels 1,

Figure 4.3 Continuous 40 s recording of MOMP channel activity in a bilayer bathed in 100 mM KCl-10 mM Tris-HCl (pH7.4) (holding potential -70 mV, filtered at 200 Hz). The closed level of the channel (0) and three open levels (1, 2 and 3) are indicated.

Figure 4.4 Amplitude histogram analysis of the 40 s continuous channel; recording illustrated in Figure 4.3.


0
1
2
3
0
1
2
3
0
1
2
3
0
1
2
3
500 ms
30 pA


12000
10000
8000
6000
4000
2000
0
Frequency
1
2
3
-40
-35
-30
-25
-20
-15
-10
Amplitude (pA)

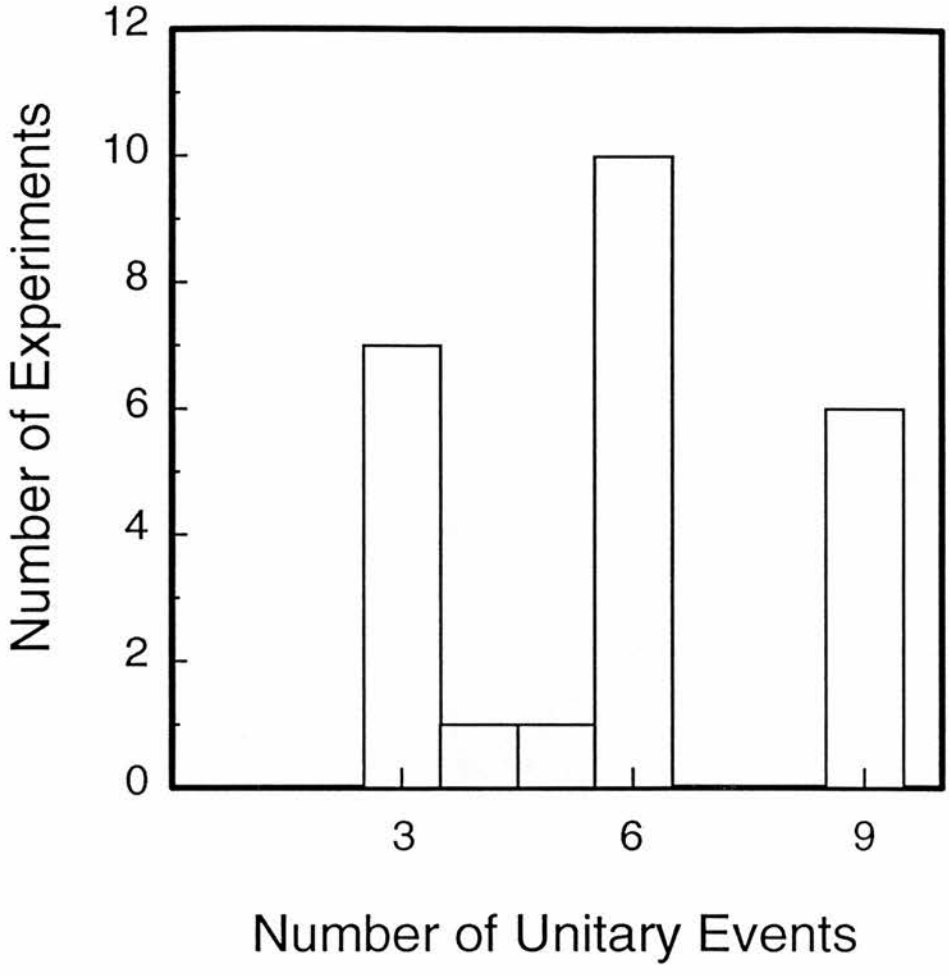


Figure 4.5 The maximum number of unit open levels counted in 25 individual experiments.

2 and 3 in Fig. 4.3) were identified and recorded while the channel was in "steady-state". Analysis by all-points amplitude histogram of individual channels consistently revealed three equally-spaced unit conductances (Fig. 4.4), suggesting the presence of a "triple barrelled" structural arrangement. The number of unit currents were counted in 25 independent experiments in which incorporated channels were maximally-activated by switching between +120 and -120 mV. The number of discrete individual conducting units are summarised in Figure 4.5 and were, invariably, grouped in multiples of three, lending weight to the 'triple barrel' hypothesis.

Unit currents were measured by amplitude histogram analysis (pClamp 6) to construct open channel current-voltage (I/V) relationships in symmetrical KCl (the same concentration in both the *cis* and *trans* chambers) (Fig. 4.6). These were all linear, the slope conductances were 120 +/- 18 pS, 210 +/- 25 pS and 310 +/- 32 pS in 50, 150 and 300 mM KCl, respectively (mean +/-SD, n = 4). The plotting of slope conductances *versus* ionic *activities* of KCl, resulted in a linear relationship which appeared to rectify at much higher ionic concentrations (Fig. 4.7). Channel recordings in concentrations of KCl above 500 mM, in order to further investigate the rectification of MOMP channel conductance, were not possible due to the unmeasurably high unit currents of channels in these ionic concentrations.

Careful inspection of the data revealed that MOMP channel closure was incomplete, with a residual conductance equivalent to approximately 5% of the fully-open state. In several MOMP channel recordings subconductance states were also observed (Fig. 4.8). These substates were markedly noisier than the fully open channel indicating unresolvably fast switching between states. However, substates, and the residual conductances of the "closed" states, were not examined in detail.

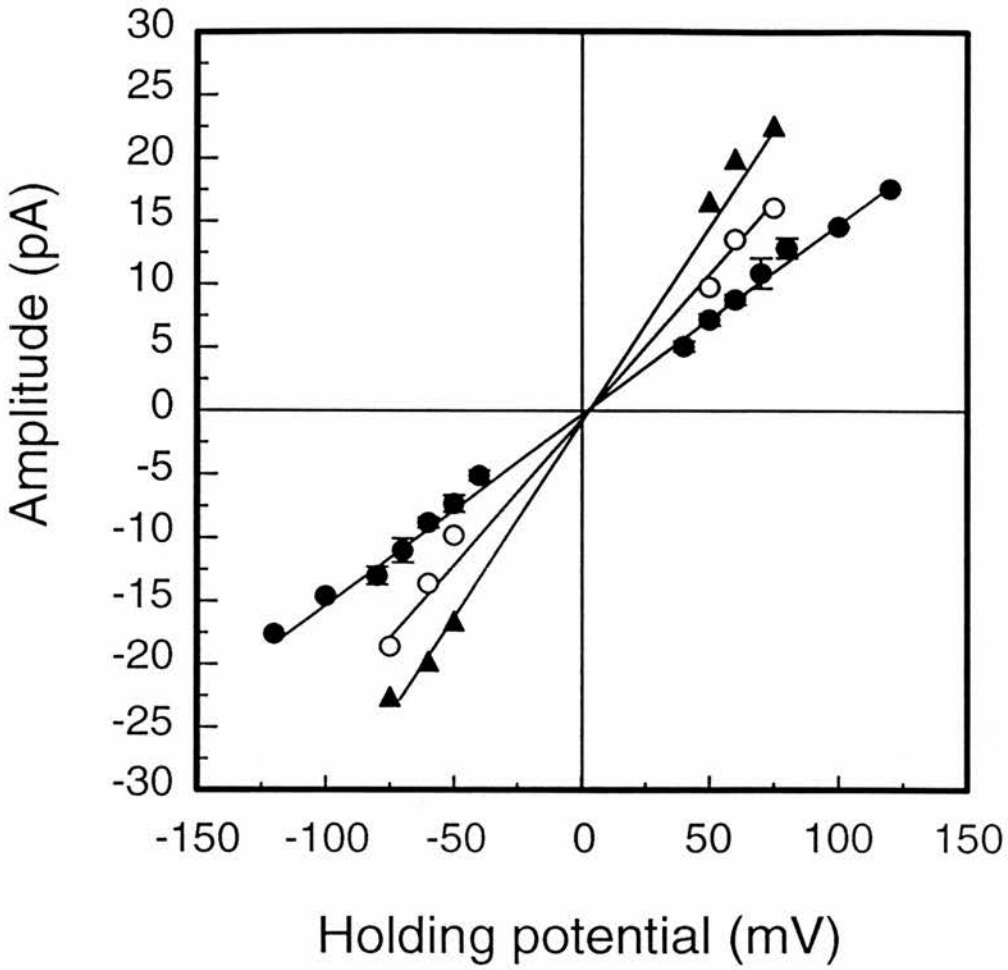


Figure 4.6 Current-voltage relationship of MOMP channels in symmetrical concentrations of KCl (each buffered with 10 mM Tris-HCl (pH7.4)). KCl concentrations are 300 mM (△), 150 mM (o) and 50 mM (•). Error bars represent +/- standard error of the mean for four independent experiments.

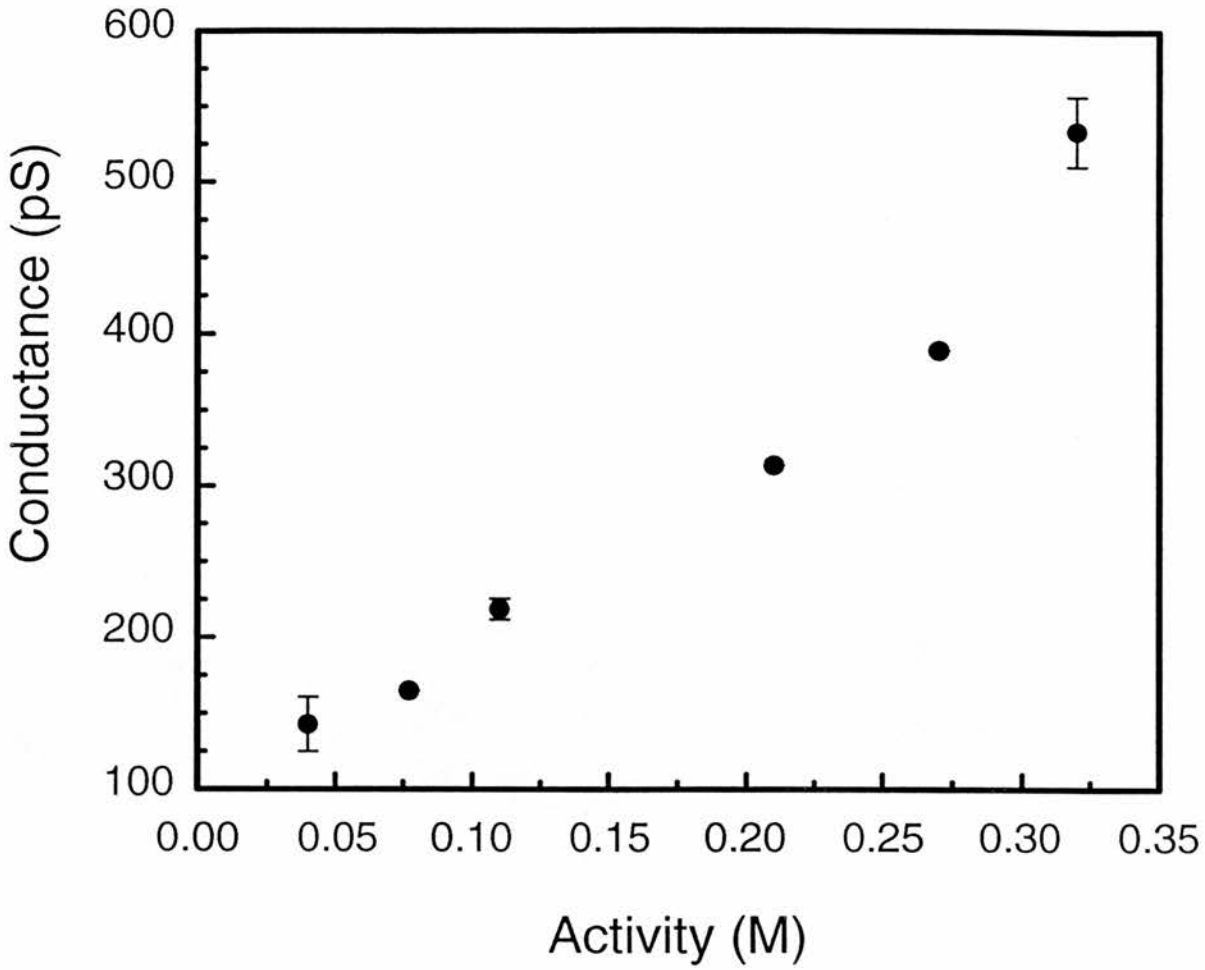


Figure 4.7 Analysis of *C. psittaci* MOMP channel slope conductance. Error bars represent +/- standard error of the mean for four independent experiments.

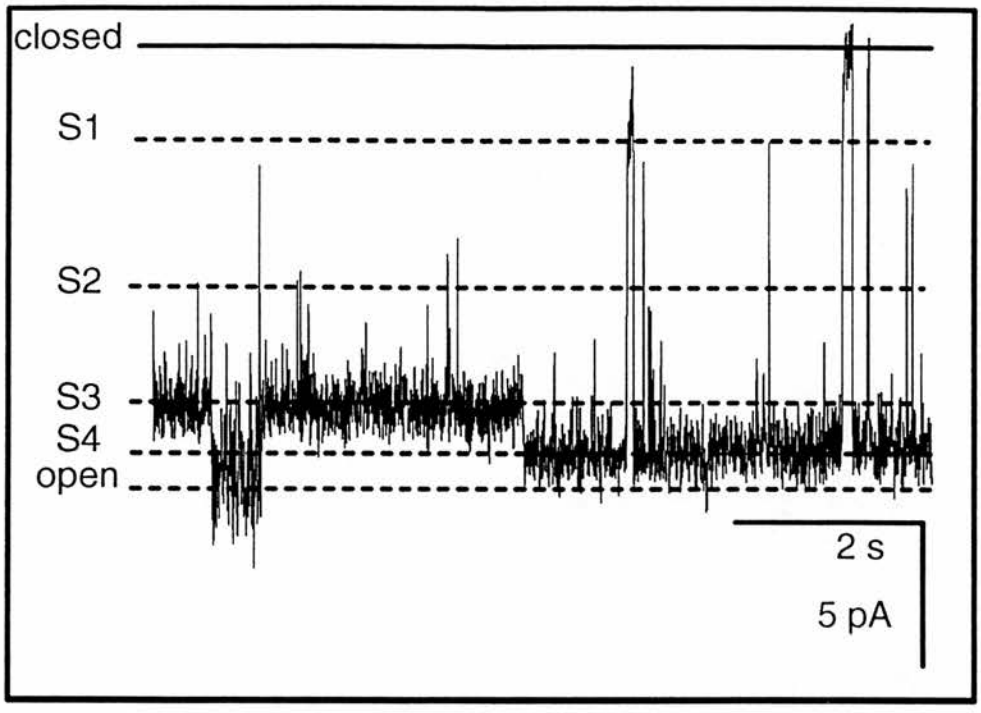


Figure 4.8 Illustration of *C. psittaci* MOMP channel subconductance states. The bilayer was bathed in 50 mM KCl-10 mM Tris-HCl (pH7.4) and the holding potential was -100 mV. This recording was low-pass filtered at 100 Hz. Channel closed level denoted by solid line and S1-4 indicates subconductance states.

Analysis of unit MOMP channels routinely takes place in solutions buffered with Tris-HCl to pH 7.4. These conditions are unlikely to mimic those experienced by MOMP in the outer membrane of phagocytosed EBs. Indeed, Schramm *et al.* (1996) directly measured the pH of *C. trachomatis*-containing vesicles and found that, despite the absence of a dramatic vesicle acidification as might have been expected, the pH of the vesicle did fall to 6. In view of these results, some channel recordings were made in KCl solutions buffered to pH 6. Both channel size (Fig. 4.9) and frequency of open/closed transitions increased significantly in 50 mM KCl (buffered with 10 mM Tris-HCl, pH 6.0). The amplitude of unit channels increased on average by 35%, in comparison to channels recorded in KCl buffered to pH 7.4.

4.2.4 Ion selectivity and permeability of MOMP channels

The relative anion *versus* cation permeability of reconstituted MOMP channels was calculated from reversal potentials (Section 2.10.2) measured in 250 mM KCl *cis versus* 50 mM KCl *trans*. A reversal potential of +10 +/- 4 mV (mean +/- SD, n = 4) (Fig. 4.10) indicated that the channel was weakly anion selective, $P_{Cl}/P_K = 2.0$ +/- 0.84 (mean +/- SD, n = 4). In addition, the channels were shown to be permeable to ATP. In the presence of 100 mM NaATP *cis versus* 10 mM NaATP *trans,* a mean reversal potential of -11 +/- 1.9 mV (mean +/- SD, n = 10 independent experiments) was obtained with a unit conductance of approximately 80 pS. The ratio of ionic *activities* 100 mM NaATP: 10 mM NaATP, measured using the Na^+ ionophore gramicidin (Gennis, 1989), was 4.5. This corresponds to a theoretical reversal potential (calculated using an adaptation of the Nernst equation) of -39 mV for a solely Na^+ selecting channel and +10 mV for a solely ATP^{4-} selecting channel, so that a reversal potential of -11 mV suggests there is substantial

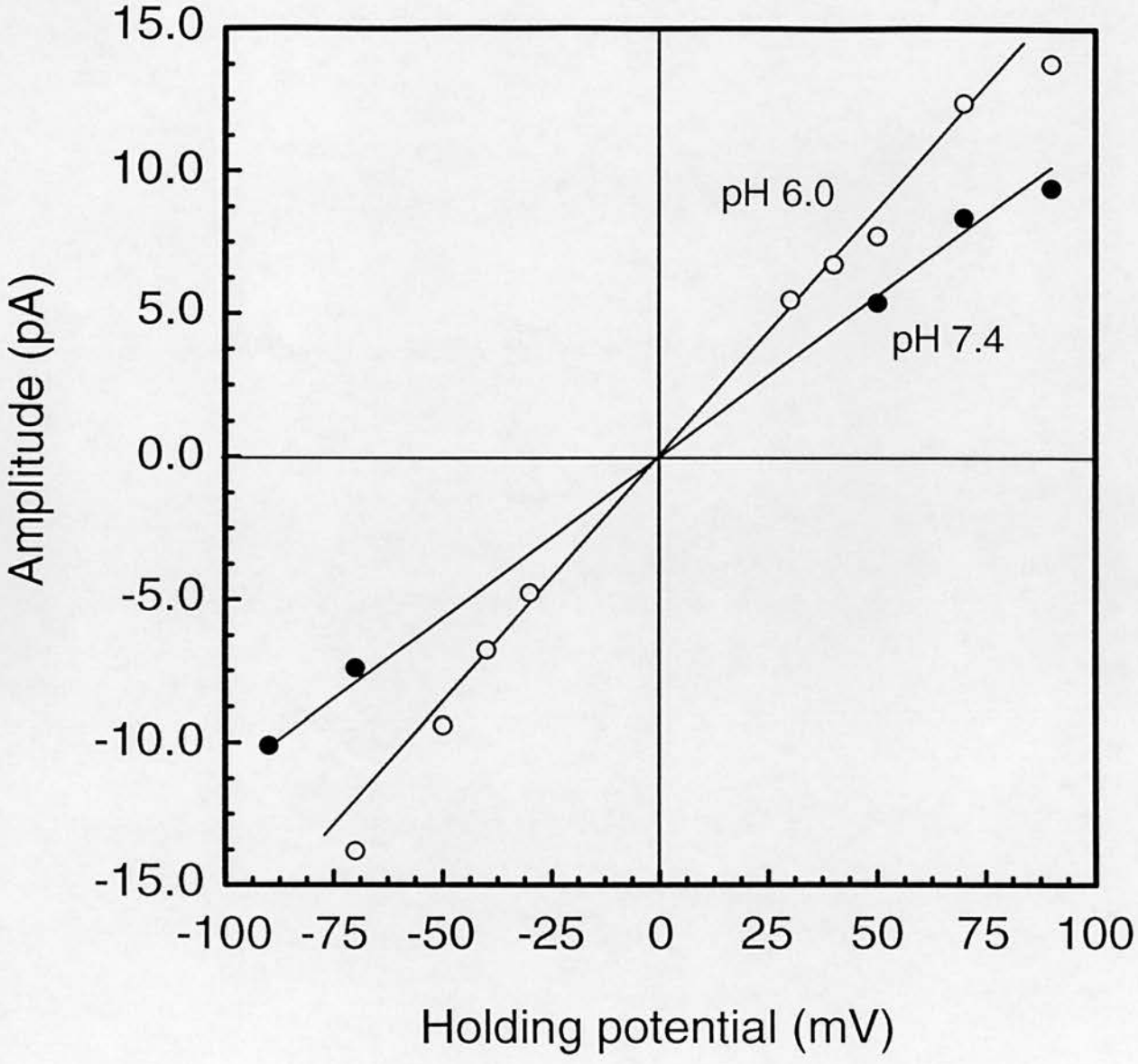


Figure 4.9 Current-voltage relationship of MOMP channels in 50 mM KCl buffered with 10 mM Tris-HCl (pH7.4) or 10 mM Tris-HCl (pH6.0) as indicated.

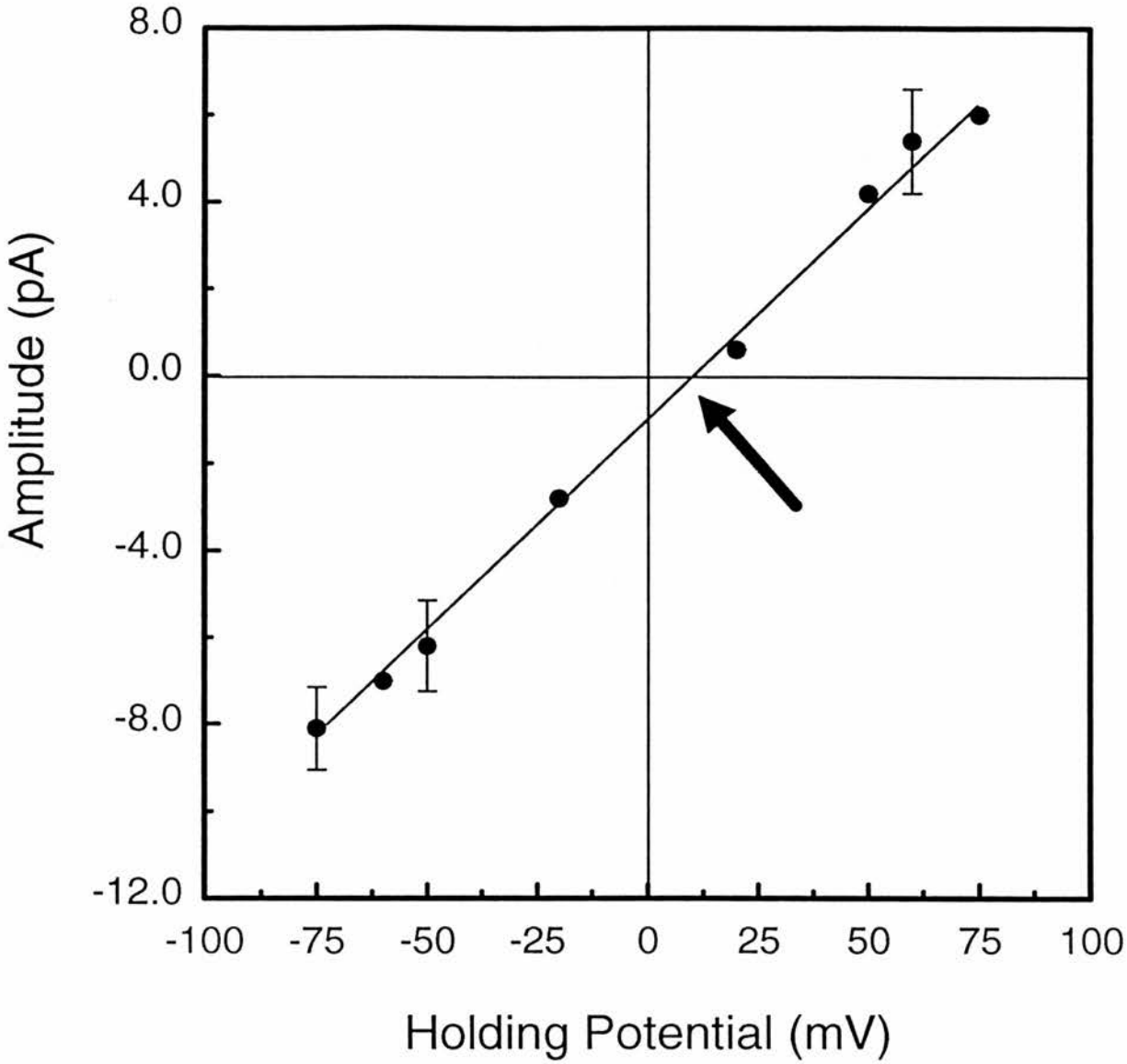


Figure 4.10 Current-voltage relationship of MOMP channels in a bilayer bathed in 250 mM KCl (*cis*) and 50 mM KCl (*trans*), each buffered with 10 mM Tris-HCl (pH7.4). Arrow indicates the reversal potential. Error bars represent +/- standard error of the mean for four independent experiments.

ATP transport.

4.2.5 *In vitro* oxidation of MOMP channels

In view of the results obtained by liposome swelling indicating that the reduction of disulphide bonds was necessary to fully open pores within the chlamydial membrane (Bavoil *et al.,* 1984), we attempted to oxidise our fully open MOMP channels and observe any effects on channel behaviour. However, *in vitro* oxidation of bilayer incorporated MOMP using increasing amounts of hydrogen peroxide, oxidised glutathione and Cu^{2+}-phenanthroline had no observable effect on any channel properties. It should be noted that continued channel activity was not dependent on the presence of DTT within buffers bathing MOMP-containing bilayers. Indeed, the addition of increasing amounts of DTT to solutions bathing bilayers incorporating functional MOMP channels, had no observable effect on any aspect of the channel characteristics.

4.3 Discussion

Using the technique of liposome swelling Bavoil *et al.* (1984). demonstrated that outer membrane complexes of *C. trachomatis* contained water-filled pores with an exclusion limit of 850-2250 Da. Due to its abundance in these outer membrane preparations, it was postulated that MOMP was responsible, as a 'chlamydial porin'. This hypothesis is amply supported by both the structural and functional results of this study.

The protocols used to purify MOMP for structural analysis (Section 3.3.2) could not be employed during the purification of MOMP for routine reconstitution experiments due to the destabilising effects of SDS (contained in both samples) on

the bilayer. Unfortunately, dialysis to remove SDS from these highly purified MOMP samples was not feasible because of the prohibitively small amounts of protein involved. However, MOMP resulting from all these methods of purification, formed SDS-resistant oligomers typical of classical gram-negative porin proteins. Indeed, limited reconstitution of both these highly purified MOMP fractions, into planar lipid bilayers, was attempted resulting in the appearance of unit channels identical in amplitude and behaviour to those observed upon bilayer incorporation of the OG-DTT solubilised sample. Not only do these results confirm that MOMP is the channel forming protein of the OG-DTT sample, they also suggest that LPS (not present in either of these highly purified MOMP fractions, as confirmed by Western blotting) is not required to stabilise or maintain the oligomeric structure of functional MOMP (Section 3.3.3). Subsequently, this has also proven to be the case with the successful reconstitution of fully functional native, recombinant MOMP channels in the absence of LPS (Chapter 5).

At a functional level, the insertion of MOMP into planar lipid bilayers seemed to be autocatalytic, resembling that seen with other porins (Xu and Colombini, 1996). It is possible that the correct insertion of one channel assisted the correct orientation of other channel proteins, thereby accelerating subsequent insertion. The preference shown by MOMP for a particular bilayer orientation was demonstrated by the asymmetric response of the channel to holding potentials of opposing polarity. Presumably this asymmetry of behaviour, also seen in other porins, has a structural basis which, if known, may explain autocatalytic insertion.

The modification of channel amplitude and open/closed transitions by the neutralising MOMP-specific mAb A11 was of particular significance. Not only did this confirm that MOMP was the channel forming protein, it also reinforced the

belief that MOMP is arranged as an oligomer in the bilayer. A11 only recognises oligomeric MOMP as judged by Western blotting (McCafferty *et al.,* 1995). This posed the question - what is the minimal conducting unit of the MOMP channel? From the results summarised in Figures 4.3 to 4.5 we suggest that each MOMP molecule inserts into the bilayer as a trimer to give rise to 3 pores through the membrane and, that switching to holding potentials of +/- 60 mV, and above, promotes the opening of all 3 pores together. Thus, 2 MOMP trimers could give rise to up to 6 pores with 6 unit currents. Voltage-dependent closure at maintained high holding potentials meant that within a few seconds, the steady-state gating of the channel resulted in only one or two "protochannels" in each trimer opening at any one time. This type of "multibarrel" behaviour is common in porins (Nakae *et al.,* 1979), and a similar phenomenon has also been observed, and analysed in more detail, in eukaryotic channel proteins (Hayman and Ashley, 1993; Clark *et al.,* 1997). It is somewhat surprising that mAb 4/11, which recognises both monomeric and oligomeric MOMP, does not have an effect on channel properties. This antibody is known to bind to the VSII of MOMP which is believed to form a surface exposed loop. The significance of this result will be discussed more fully in Chapter 7.

As demonstrated by Schramm *et al.* (1996), the pH of *Chlamydia*-laden inclusions does not fall below 6 for up to 12 hours post infection. However, this small drop in pH from 7 to 6 has been suggested to be sufficient to catalyse the conversion of EBs to RBs. As MOMP is almost certain to function as a porin within such an inclusion, properties of the MOMP channel were assessed in equivalent acid conditions. Alteration of the buffer pH from 7.4 to 6.0 was sufficient to increase unit current amplitude by an average of 35%. In addition,

open/closed transitions occured more frequently. These results may be the result of a re-arrangement of charged groups in the mouth of the MOMP channel due to the increased environment of H^+. Indeed, they suggest that MOMP only becomes fully open within the mildly acidified inclusion.

What ions and metabolites would be translocated across the chlamydial outer membrane by a MOMP porin? Previously, *Chlamydia* were thought to require host-derived nucleotide triphosphates as an energy source and precursor of RNA synthesis, prompting us to look at their transfer across the bilayer. The reversal potential of -11 mV observed in asymmetric concentrations of NaATP corresponded to the passage of substantial amounts of ATP across the bilayer. If the MOMP channel was Na^+-selective, allowing only the passage of Na^+, we would theoretically expect to see a reversal potential of -39 mV, alternatively if the channel was only allowing the passage of ATP^{4-} across the bilayer, a reversal potential of +10 mV would be observed. Therefore a reversal potential of -11 mV demonstrates the passage of both Na^+ and ATP^{4-} through the MOMP channel. The identification of a route by which *Chlamydia* can take advantage of host nucleotide triphosphates and other nutrients may also explain why antibodies specific to MOMP neutralise infection. However, it now appears unlikely that *Chlamydia* derive all their ATP from the host cell with the identification of genes encoding ATP biosynthetic pathways by the *Chlamydia* Genome Sequencing Project (Stephens *et al.,* 1998). It may be the case that chlamydiae require host-derived nucleotide triphosphates to kick start their own metabolism, alternatively, in times of nutrient stress within the host cell the bacteria may then resort to the synthesis of its own ATP. This area needs further investigation.

In the EB, outer membrane proteins are disulphide bond cross-linked, rendering the outer membrane largely impermeable. The transition into the intracellular RB is coupled with a reduction of outer membrane disulphide bonds, increasing membrane permeability. It is clear that MOMP's pore forming activity would be primarily utilised at the RB stage of the chlamydial lifecycle. Bavoil *et al.* (1984) proposed that the reduction of outer membrane disulphide bonds "opened" chlamydial pores, allowing the uptake of ATP and other nutrients. Evidence supporting this hypothesis included activation of the pores by treatment with DTT, and the blocking of re-oxidation with iodoacetamide. Chemical modification was not required to maintain functional 'open' channels from the OG-DTT-solubilised MOMP. We also noted that we could not re-oxidise the putative free SH groups of bilayer incorporated MOMP to close or inactivate the channels (mimicking the EB to RB transition). However, the re-oxidation of disulphide bonds in the correct pairings to close the channel may require the presence of other proteins, such as the cysteine rich proteins absent from the OG-DTT-solubilised sample.

In this study we have shown that MOMP functions as a porin, and have demonstrated a possible physiological function with the passage of nucleotide triphosphates through the channel. A particularly significant aspect of the work is that it provides a measurable criterion by which to assess recombinant MOMP for native function (Chapter 5). These results have broad implications on the search for an effective chlamydial vaccine.

CHAPTER FIVE

RESULTS:

STRUCTURAL AND FUNCTIONAL ANALYSIS OF *CHLAMYDIA PSITTACI* AND *CHLAMYDIA PNEUMONIAE* RECOMBINANT MOMPS

5.1 Introduction

Due to the difficulties and expense inherent in the production of large amounts of chlamydial antigen, the best approaches to vaccine development rely on the production of recombinant antigen. In particular, research into the design of MOMP sub-unit vaccines has been based on the use of highly immunogenic oligopeptides and cloned recombinant fragments (Su and Caldwell, 1992; Murdin *et al.,* 1993; Zhong *et al.,* 1994). The success of these vaccines has been disappointing, perhaps because these immunogens do not induce protective cellular and humoral immune responses elicited by natural infection with the organism. This suggests that a successful vaccine may need conformational information in order to elicit a long-lasting protective immune response.

Unfortunately, previous attempts to express functional, native MOMP in heterologous expression systems have failed. Gram-negative bacteria such as *E. coli* synthesise OMPs within the cytoplasm as precursor proteins with N-terminal signal sequences. These precursor proteins are then translocated across the bacterial inner membrane where the signal sequence is cleaved and are then sorted to the outer membrane by one of two proposed pathways. The OMPs may be directly inserted into the outer membrane via contact points between the two membranes (Bayer *et al.,* 1982). Alternatively, with the aid of periplasmic molecular chaperones, OMPs may form folded intermediates within the periplasm before being inserted into the outer membrane (Nikaido, 1994; Tommassen and de Cock, 1995). Almost without exception, recombinant MOMP expression results in protein accumulating in the *E. coli* periplasm as an inclusion body. The exact reason for this is unknown. Chlamydial signal sequences may not be recognised by *E. coli* and

therefore signal sequence cleavage, following translocation across the inner membrane, may not take place. It is possible that the retention of the signal sequence may result in the aberrant folding of recombinant MOMP within the periplasm thus preventing its transport to the outer membrane. Alternatively, accumulation in the periplasm may be a consequence of the highly disulphide bond cross-linked structure of MOMP. Incorrect disulphide bond pairings formed in the periplasm may also lead to the misfolding of recombinant MOMP, resulting in its accumulation and aggregation as an inclusion body.

It is clear that the ability to express recombinant MOMP and refold to native conformation would have broad implications on the search for an effective chlamydial vaccine. Indeed, the planar bilayer system used to demonstrate the porin-like function of reconstituted native MOMP may provide a measurable criterion by which to assess recombinant MOMP for native function.

5.2. Structural and functional analysis of recombinant *C. psittaci* MOMP expressed in a cell-free transcription/translation coupled system

In view of the difficulties in expressing recombinant chlamydial MOMP in *E. coli* cells (Section 5.1), expression in an alternative cell-free system was attempted. The *E. coli* T7 S30 extract system for circular DNA was used for the coupled transcription/translation of the truncated *C. psittaci* MOMP construct, tMOMP (Section 2.8.2). This cell-free system contained all the necessary components for transcription and translation. 3 μg of tMOMP DNA was used, and translated proteins were radioactively labelled by adding [^{35}S] L-methionine to the reaction mixture. Recombinant *C. psittaci* MOMP expressed using the *E. coli* T7

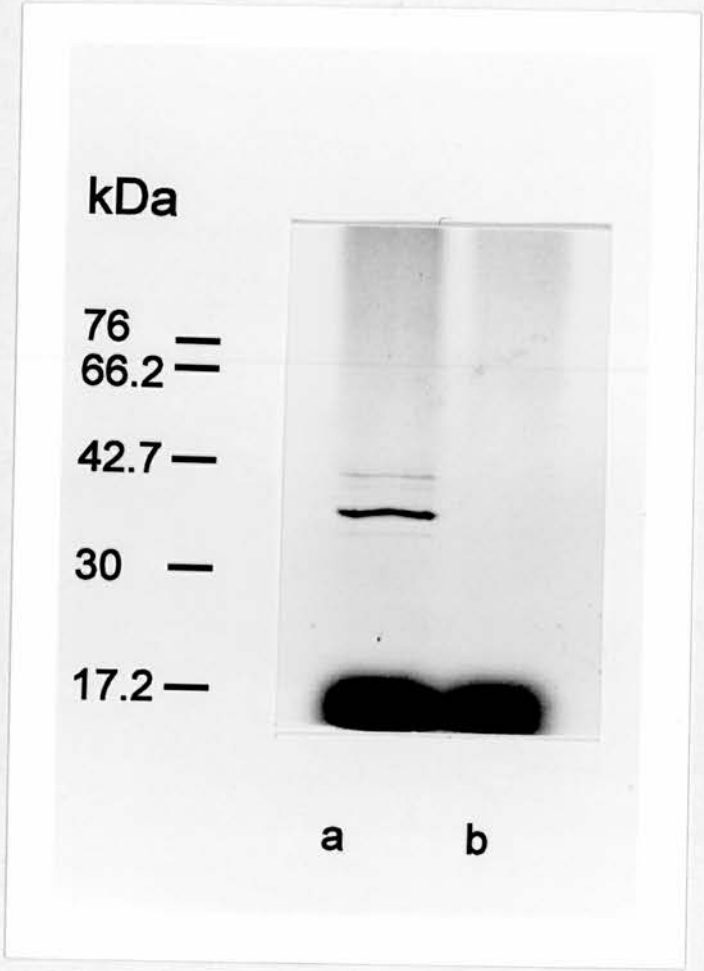


Figure 5.1 SDS-PAGE and Western blot analyses of *C. psittaci* tMOMP expressed in the *E. coli* T7 S30 *in vitro* transcription/translation system. Radioactively labelled, expressed protein (a) and uninduced T7 reaction mixture (b) were subjected to SDS-PAGE analysis on 12.5% gels, blotted onto nitrocellulose membrane and developed onto film by autoradiography.

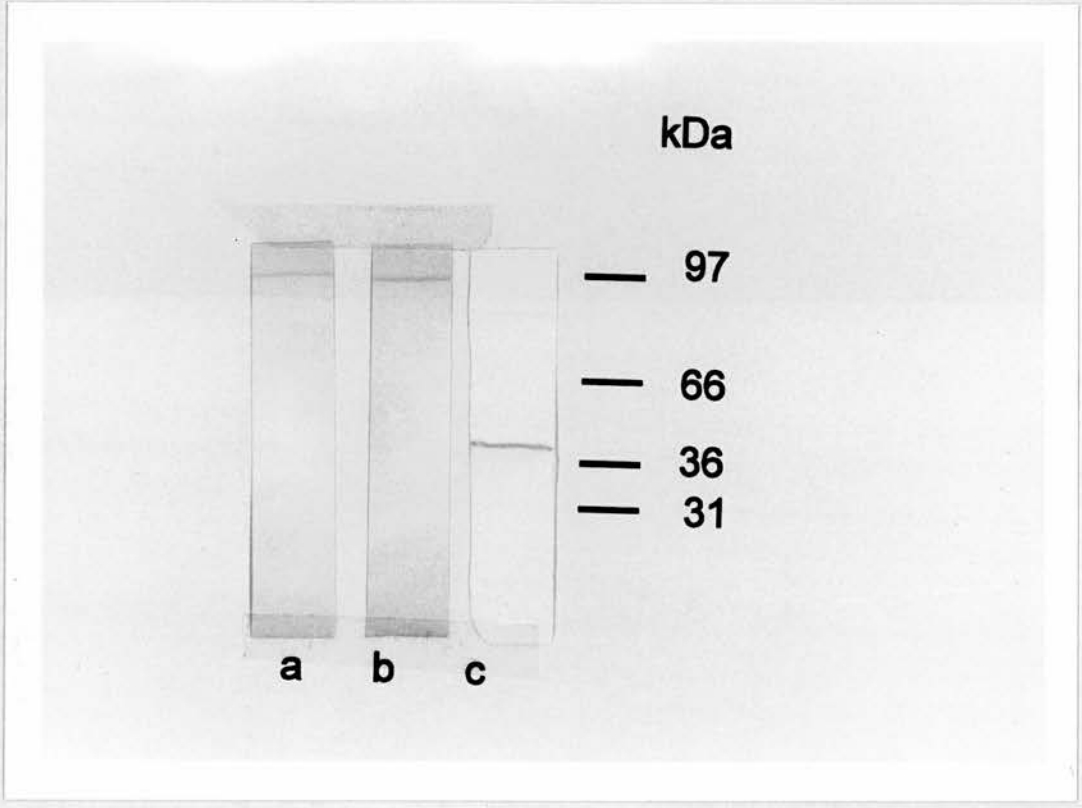


Figure 5.2 Western blot analysis of *C. psittaci* tMOMP expressed in the *E. coli* T7 S30 extract system. Expressed protein was run on 12.5% gels, immunoblotted, and probed with mAb A11 (sample loaded without boiling, lane a) and mAb 4/11 (sample loaded without boiling, lane b; and after boiling, lane c).

S30 extract system migrated as a single band with an apparent molecular mass of 37 kDa when analysed by SDS-PAGE after denaturing by boiling in SDS-sample buffer in the presence of a reducing agent (Fig. 5.1). Three μg of tMOMP DNA yielded approximately 35 μg/ml of protein, as estimated by densitometry. Western blot analysis showed that the 37 kDa protein was recognised by MOMP-specific mAb 4/11. In contrast, SDS-PAGE analysis of cell-free expressed tMOMP, in SDS-sample buffer (and reducing agent) but not fully denatured by boiling, revealed a protein band with a molecular mass of 100 kDa. This band was interpreted to be an oligomer of MOMP and was found to react with native MOMP-specific mAb A11 upon immunoblotting (Fig. 5.2).

Planar lipid bilayer reconstitution of cell free expressed tMOMP resulted in the appearance of unit channels identical in amplitude and behaviour to those observed upon incorporation of the OG-DTT solubilised native MOMP (results not shown). Unfortunately, this recombinant MOMP sample could not be routinely reconstituted into the bilayer as the *E. coli* T7 S30 extract system reaction mixture contained unknown detergents which destabilised the bilayer. No channel activity was observed following the reconstitution of the uninduced *E. coli* T7 S30 reaction mixture.

5.3 Cloning, expression and analysis of recombinant MOMP from *C. psittaci* and *C. pneumoniae*

5.3.1. *C. psittaci* and *C. pneumoniae* recombinant MOMP cloning

The *C. psittaci* recombinant MOMP construct used in this study was engineered previously at the Moredun Research Institute (Herring *et al.*, 1998). It encodes a truncated version of MOMP (termed tMOMP) starting at the first natural

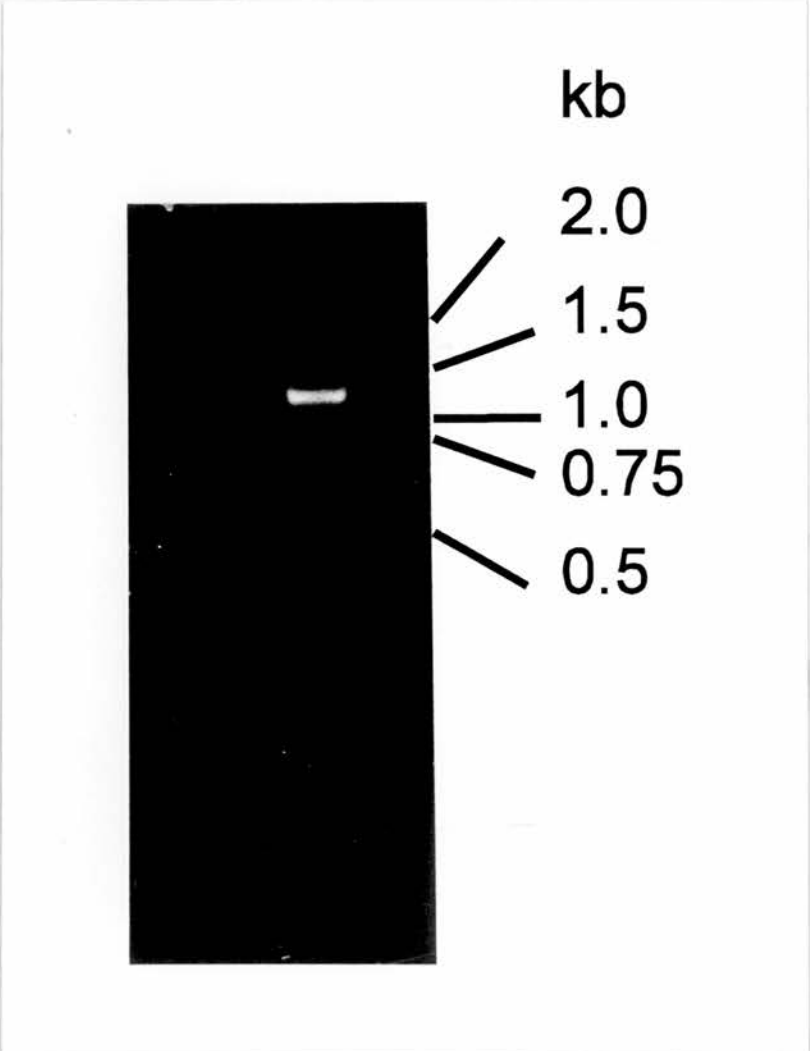


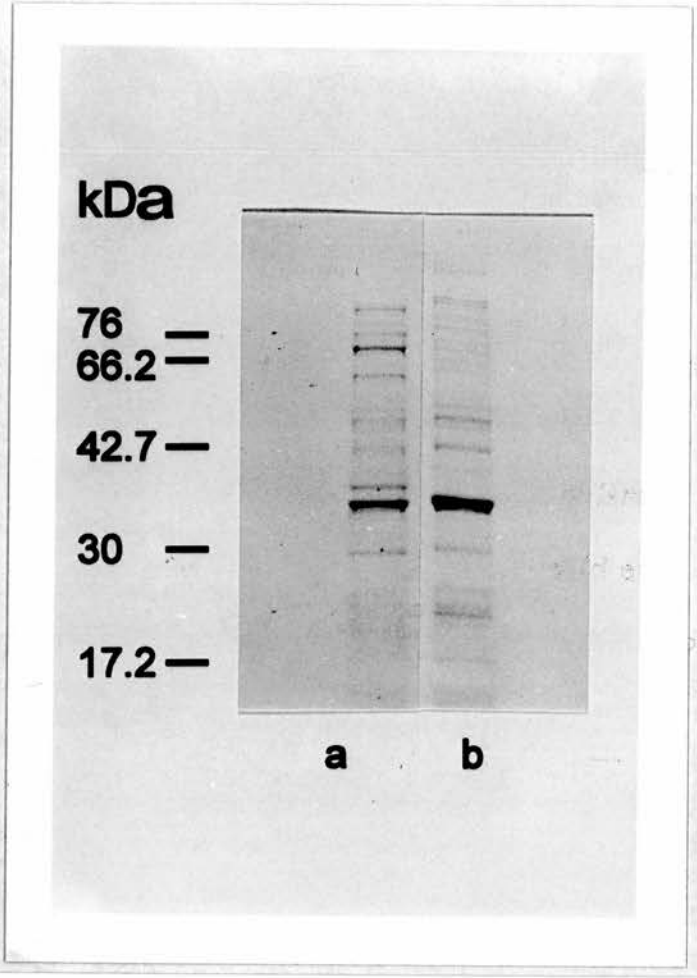
Figure 5.3 PCR product amplified from *C. pneumoniae* strain IOL207 genomic DNA. Analysed on 2% (w/v) agarose gels with ethidium bromide.

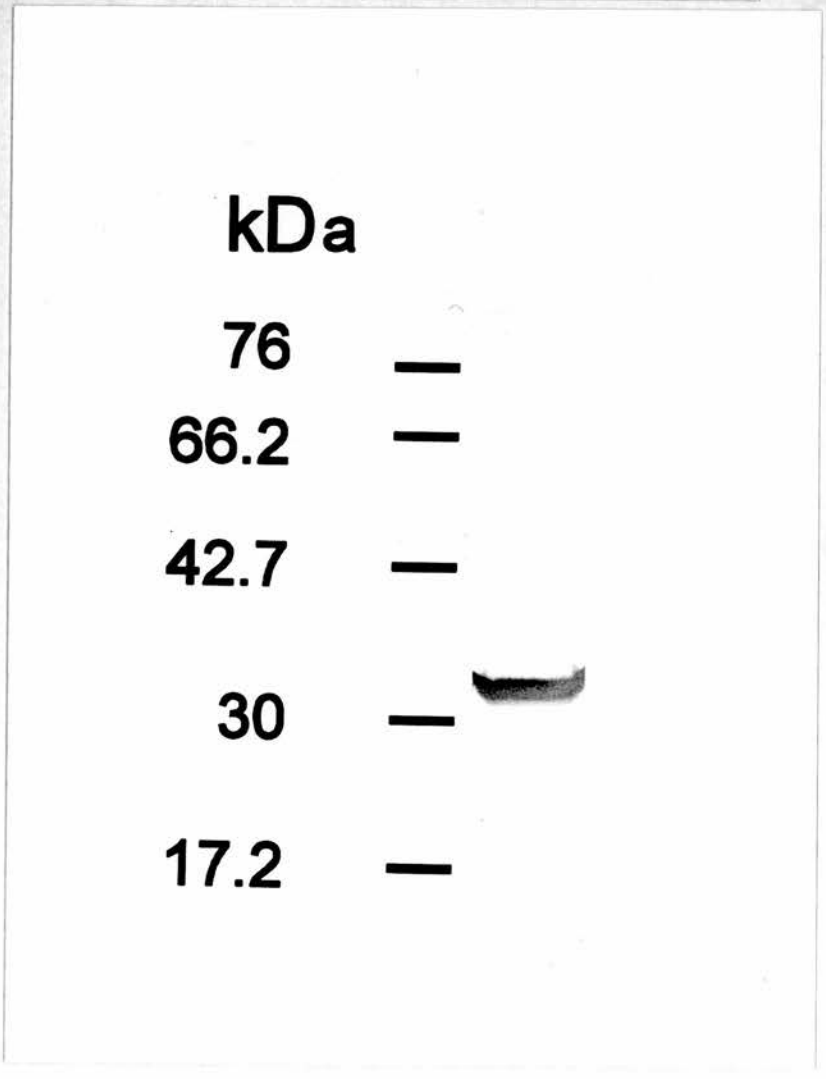
methionine, thus does not encode the first 16 amino acids of the mature protein. *C. pneumoniae* tMOMP was engineered in a similar way.

The *C. pneumoniae* MOMP gene, amplified from *C. pneumoniae* IOL-207 genomic DNA, resulted in a 1.36 kb PCR fragment (Fig. 5.3) which was sub-cloned into pGEM-T. The MOMP gene fragment was subsequently cloned into the expression vector pET-22b (+) via restriction endonuclease sites *Nde*I and *Not*I. Constructs were checked before transformation into *E. coli* expression host BL21 (DE3) and induced. Expressed products were determined to form inclusion bodies which were isolated by a sonication and differential centrifugation procedure.

5.3.2. SDS-PAGE and immunoblot analysis of recombinant tMOMP

Inclusion body preparations of both the *C. psittaci* and *C. pneumoniae* recombinant MOMPs routinely yield 10-50 mg/L of protein. SDS-PAGE analysis of the recombinant proteins is shown in Figure 5.4. Both recombinant proteins migrated with an apparent molecular mass of 37 kDa when solubilised in SDS-sample buffer (including reducing agent) and denatured by boiling. Recombinant *C. psittaci* MOMP was recognised by MOMP-specific mAb 4/11 following immunoblotting (Fig. 5.5), however, this denatured protein did not react when probed with native *C. psittaci* MOMP-specific mAb A11 (results not shown). Indeed, a commercially available native *C. pneumoniae* MOMP-specific mAb also failed to recognise the denatured recombinant *C. pneumoniae* MOMP (results not shown).


kDa
76
66.2
42.7
30
17.2
a
b


kDa
76
66.2
42.7
30
17.2

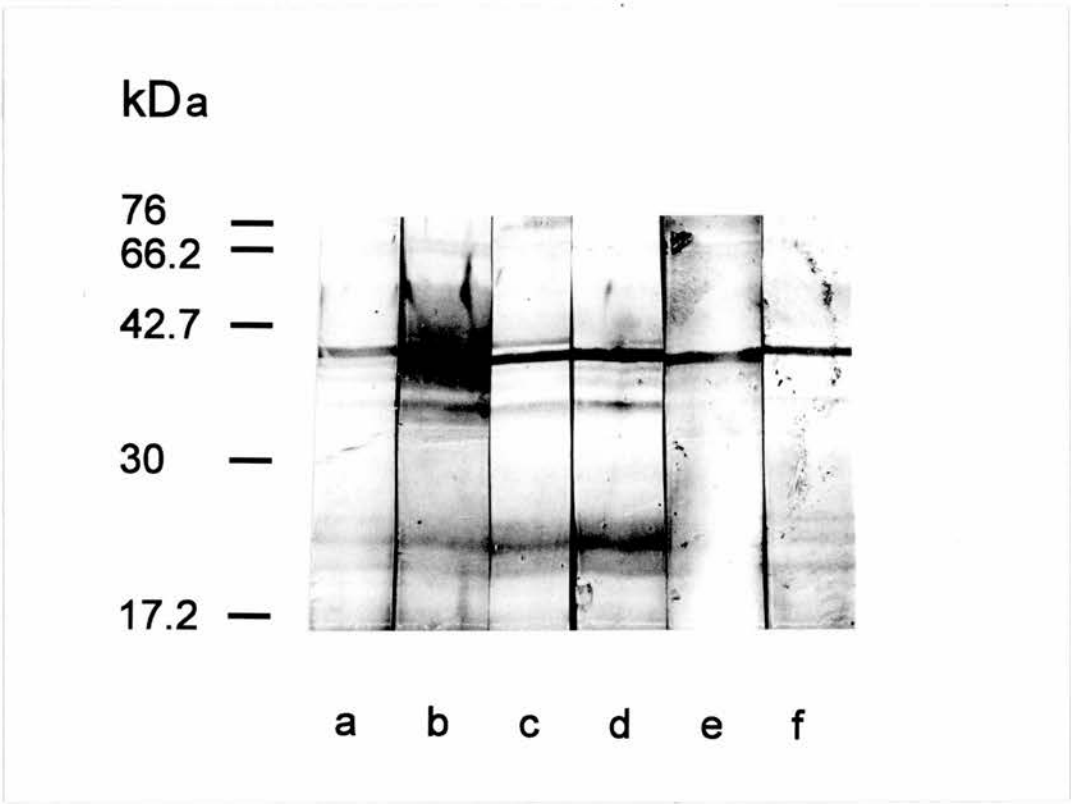


Figure 5.6 Western blot analysis of recombinant tMOMP from *C. pneumoniae*. tMOMP was subjected to SDS-PAGE on 12.5% gels, immunoblotted, and probed with anti-sera raised against the recombinant protein in rabbits (a to c) and guinea pigs (d to f).

5.3.3 *C. pneumoniae* recombinant tMOMP-specific antisera

Due to the failure of the commercially available native *C. pneumoniae* MOMP-specific mAb to recognise denatured recombinant tMOMP by immunoblotting, specific anti-sera was raised against this protein. Rabbits and guinea pigs were inoculated with the purified recombinant protein (Section 2.9) and at 3 weeks post final vaccination were bled out and sera prepared. Immunoblots of the recombinant tMOMP were probed with the sera from each animal, at final dilution of 1/500. A 37 kDa protein, corresponding to recombinant *C. pneumoniae* tMOMP, was strongly recognised by sera from all animals (Fig. 5.6). Pre-inoculation sera from these animals showed no reaction with the recombinant protein.

5.4 Planar lipid bilayer analysis of recombinant MOMP

5.4.1. Reconstitution of *C. psittaci* and *C. pneumoniae* recombinant MOMPs

C. psittaci and *C. pneumoniae* OG/DTT-solubilised tMOMPs were introduced to the *cis* chamber of the bilayer set-up in the presence of a 500 mM KCl *cis versus* 50 mM KCl *trans* gradient. Under these conditions, recombinant MOMP incorporated into the bilayer within 5-10 min of addition, to give rise to ion-channel-like unit conductances. After the first evidence of channel incorporation, the *cis* chamber was perfused with a minimum of 10 volumes of 50 mM KCl to limit the bilayer channel content. However, channel incorporation appeared to be autocatalytic, as previously described for native *C. psittaci* MOMP (Section 4.2.1), and as a result further channel incorporation was not uncommon. The addition of similar volumes of OG/DTT alone had no effect on the bilayer.

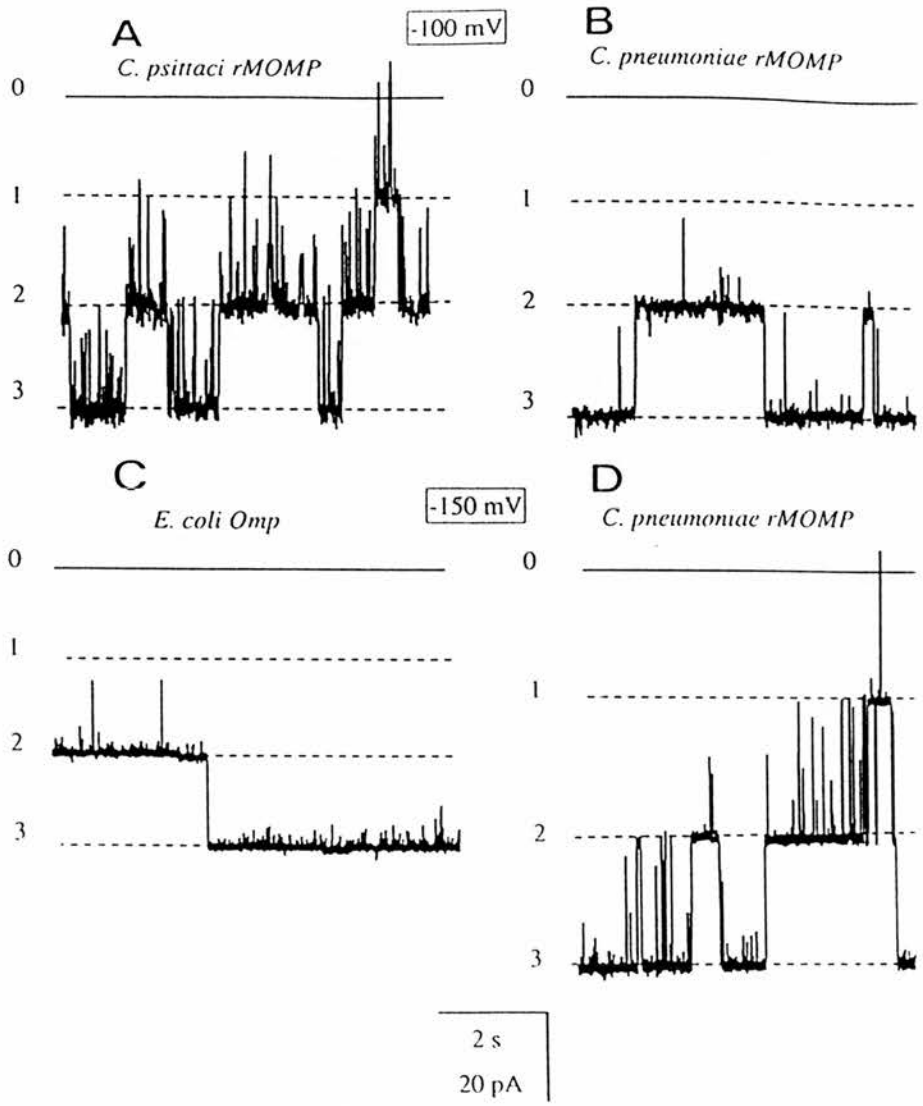


Figure 5.7 Comparison of recombinant *C. psittaci* and *C. pneumoniae* MOMPs, and the *E. coli* Omp proteins. Representative recordings of planar lipid bilayers containing the indicated proteins in 300 mM KCl, 10 mM Tris-HCl, pH7.4, at a holding potential (*cis* minus *trans*) of either -100mV (A, B) or -150 mV (C, D). The dotted lines indicate unit current open levels (openings downwards at negative potentials). Filtered at 100 Hz.

5.4.2. Single channel recordings of recombinant tMOMPs

The single channel properties and gating behaviour of the recombinant MOMP channels were studied in detail. Figure 5.7 shows representative ion-channel recordings resulting from the bilayer incorporation of recombinant *C. psittaci* (A) and *C. pneumoniae* (B, D) MOMPs, and native *E. coli* Omps (C). The planar bilayer was voltage clamped at -100 mV or -150 mV as indicated (*cis* minus *trans*) as indicated, and bathed in 300 mM KCl containing 10 mM Tris-HCl (pH 7.4). Both *C. psittaci* and *C. pneumoniae* recombinant MOMPs routinely opened to three distinct levels (1-3, as indicated in Fig. 5.7). This apparent trimeric behaviour mimics that previously observed with native *C. psittaci* MOMP porin (Section 4.2.3). The threshold potential required to close the *C. psittaci* recombinant channel was higher than that required to close the native channel (about -100 mV compared to -80 mV) (Section 4.2.1). The gating pattern (and unit amplitude) of the recombinant *C. pneumoniae* MOMP channel was clearly different from that of *C. psittaci*, and this porin required an even higher threshold potential of -150 mV to bring about significant channel closure.

5.4.3. Comparison with *E. coli* Omps

In order to rule out the possibility that *E. coli* porins had contaminated *C. psittaci* and *C. pneumoniae* MOMP preparations, outer membrane proteins of the *E. coli*-type used to express these recombinants were solubilised in OG/DTT (Section 2.4.1) and reconstituted into bilayers. These *E. coli* porins displayed very different channel properties to those of the chlamydial porins (Fig. 5.7 and Table 2). The native *E.coli* Omps had a substantially lower conductance than the chlamydial MOMPs, while requiring a significantly higher threshold potential to

	[KCl] (mM)	*C. psittaci* MOMP		*C. pneumoniae* MOMP	*E. coli* Omp
	(*cis*/*trans*)	native	recombinant	recombinant	native
g (pS)	50/50	120 ± 18 (4)	130 ± 8 (3)^{NS}	149 ± 25 (4)*	82 ± 8 (4)***
	150/150	210 ± 25 (4)	230 ± 25 (3) ^{NS}	255 ± 21 (4)**	nd
	300/300	320 ± 32 (4)	340 ± 10 (3) ^{NS}	408 ± 0.07 (4)***	nd
$P_{Cl/K}$	250/50	2.0 ± 0.8 (4)	0.38 ± 0.2 (3)***	0.49 ± 0.07 (4)***	0.28 ± 0.02 (4)***

result in significant channel closure. Furthermore, these channels were observed to be more selective for cations than either of the recombinant MOMPs (see below and Table 2). The Omp-like channels were never observed during any reconstitution of the MOMP inclusion body proteins, and MOMP-like channels could not be reconstituted from non-induced bacteria. This work was completed in collaboration with R. H. Ashley.

5.4.4. Ion selectivity and conductance.

Comparison of the unit channel amplitudes of recombinant *C. pneumoniae* and *C. psittaci* MOMPs revealed significant differences, summarised in Table 2. The conductances of recombinant *C. pneumoniae* channels, measured over a range of KCl concentrations, were substantially higher than those of both recombinant and native *C. psittaci* MOMP. However, conductances of recombinant *C. psittaci* MOMP unit channels were not significantly different from the results reported for the native channel protein. There was a significant shift in relative anion *versus* cation selectivity between the *C. psittaci* native and recombinant porins. Both recombinant channels were weakly cation-selective, while the native protein was weakly anion selective.

5.4.5. Effect of oligomer-specific monoclonal antibody A11 on recombinant *C. psittaci* MOMP channels

The recombinant *C. psittaci* porin exhibited the same asymmetric response to holding potentials of opposing polarity as reported for the native protein (Section 4.2.1). Note the increase in 'flickery' closures for recombinant *C. psittaci* MOMP at negative (Fig. 5.7 A) compared to positive (Fig. 5.8 (control)) holding potentials.

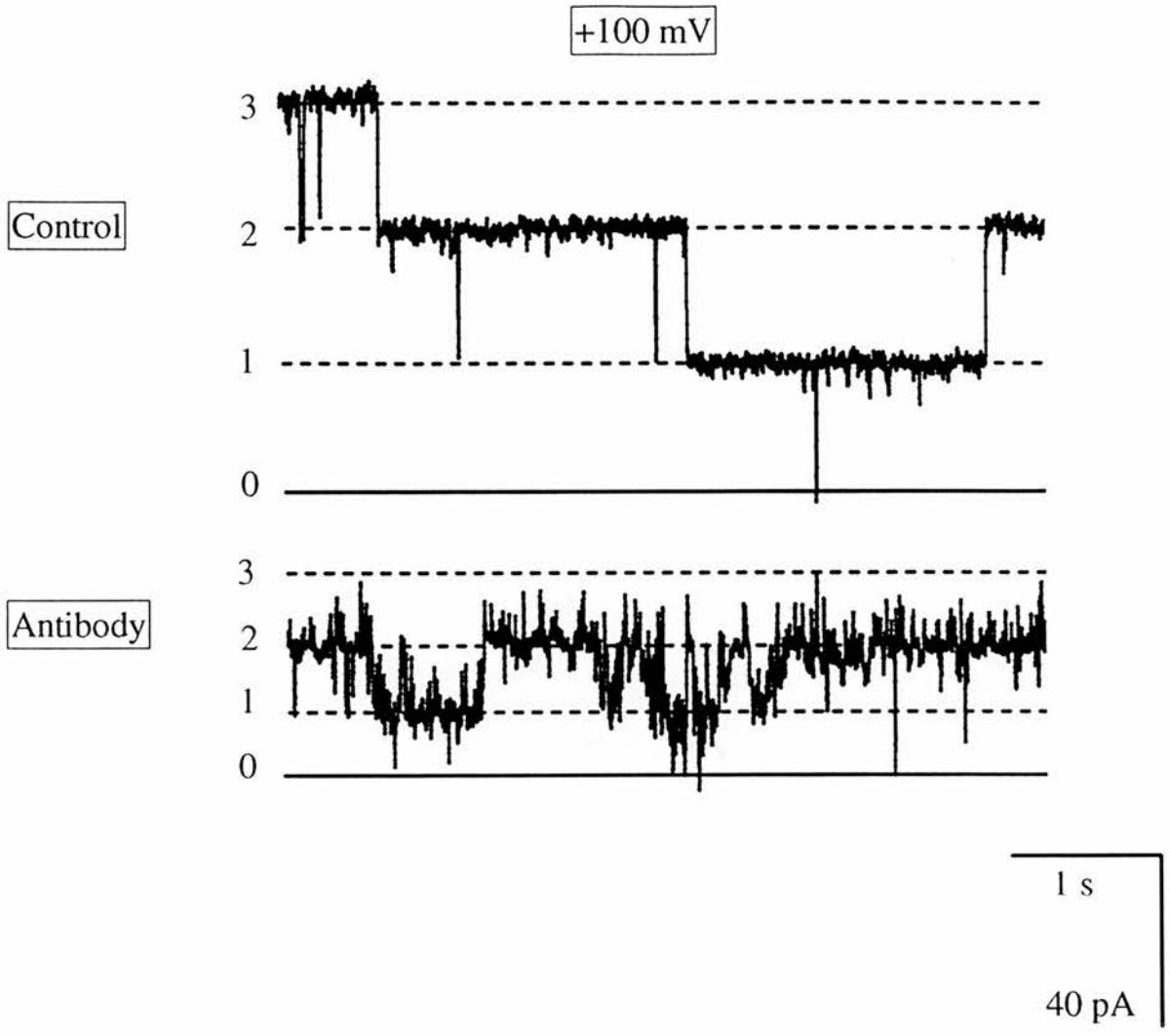


Figure 5.8 Effect of native MOMP-specific mAb A11 on recombinant *C. psittaci* channels. Bilayer bathed in 300 mM KCl, 10 mM Tris-HCl, pH7.4. mAb A11 added to both the *cis* and *trans* chambers to a final dilution of 1/500 in the presence of 1 mg/ml BSA. Note the difference in amplitude and the reduction in the number of long lived channel closures. The solid line represents the baseline level, corresponding to the closure of the unit conductance. Filtered at 100 Hz.

Addition of a native *C. psittaci* MOMP-specific neutralising mAb, A11, affected both the gating of the recombinant channel and the amplitude of channel unit conductances. Figure 5.8 shows a recording of channel behaviour before (upper trace) and 2 min after the addition of 0.25% (v/v) mAb A11 to both the *cis* and *trans* chambers (lower trace). The unit conductance amplitudes were significantly reduced, and the unit conductances showed more frequent 'flickery' closures than in the control trace. This antibody effect is very similar to that observed upon the addition of mAb A11 to the native MOMP channel (Section 4.2.2). This work was completed in collaboration with R. H. Ashley.

5.4.6. Effect of MOMP-specific antibodies on recombinant *C. pneumoniae* MOMP channels

The addition of the commercially available *C. pneumoniae* native MOMP-specific mAb had no discernible effect on the behaviour or properties of *C. pneumoniae* tMOMP channels. Furthermore, tMOMP specific anti-sera (Section 5.3.3) failed to effect these recombinant channels when added to bilayer chambers at a dilution of 1/250.

5.5. Discussion

The results presented in this chapter demonstrate the first successful expression and functional reconstitution of recombinant MOMPs from *Chlamydia* spp. We have shown that the recombinant *C. psittaci* protein is functionally similar to the previously characterised native protein, in that it exhibits similar 'triple-barrelled' behaviour and has similar conductance and gating behaviour. Reconstitution of *C. pneumoniae* recombinant MOMP demonstrates that it has

comparable conductance and selectivity properties to those of *C. psittaci* MOMP, but a higher voltage threshold for gating. Secondary structural information (Section 3.3.2) and confirmation of the ion channel function of chlamydial MOMPs (Section 4.2.1) places these proteins firmly in the porin superfamily.

The channel gating characteristics, unit channel amplitudes and selectivity of the chlamydial porins were vastly different from those of the *E. coli* Omp-like channels purified from the non-induced bacteria. In addition, the amplitude and gating of recombinant *C. psittaci* MOMP channels was modified by the addition of native MOMP-specific mAb A11. Not only does this result confirm that channel activity results from MOMP reconstitution and not contaminant *E. coli* Omps, it also demonstrates that recombinant MOMP is refolding to a functionally native conformation. The addition of a commercially supplied mAb, reportedly *C. pneumoniae* MOMP specific, had no effect on our recombinant *C. pneumoniae* channels. In contrast to the MOMPs of the other chlamydial species, *C. pneumoniae* MOMP appears to be less immunogenically and antigenically complex (Section 1.8.3) (Campbell, *et al.,* 1990; Black, *et al.,* 1991). Indeed, surface exposure of this MOMP has not been determined leading some researchers to hypothesise that it lies beneath a layer formed by other chlamydial antigens (Christiansen *et al.*, 1998). In view of these results there must be some doubt over the specificity of this antibody which has been raised against *C. pneumoniae* EBs. Similarly, antisera specific to the recombinant protein also failed to have any effect on the channel properties. These sera are likely to recognise linear epitopes on the recombinant protein which may not be surface exposed when it refolds to native conformation. This result mirrors that seen when mAb 4/11, specific to a linear epitope in the VS2 region of the native protein, failed to effect native MOMP

channel function, while mAb A11 which recognises a conformational epitope dramatically changed channel behaviour and characteristics. There is little doubt that the porin-like ion channels resulting from reconstitution of solubilised inclusion body preparations are recombinant MOMP channels with similar characteristics to those observed with *C. psittaci* MOMP.

The crystal structure of several porins have been determined (Cowan *et al.*, 1992) and without exception these proteins have been found to form β-barrels composed of 16 anti-parallel β-strands. Porin β-barrels have short intracellular turns and longer external loops, one of which is an 'eyelet', a pore-confined loop that greatly limits single channel conductance. Without this partial obstruction, single channel conductance of porin β-barrels would be an order of magnitude higher (Hille, 1992), yet conductance of MOMP is similar to that of the general diffusion porins. We therefore speculate that chlamydial porins have a similar structure. The difference in ion selectivity between *C. psittaci* recombinant and native MOMP may be due to the 16 amino acid truncation of the recombinant protein. If MOMP is structurally similar to other porins, as we suggest, this truncation could result in the loss of 2 anti-parallel β-strands. This in turn would result in the β-barrels having a slightly reduced diameter, and more importantly, the surface loops, and therefore the channel mouth and eyelet region being arranged in a slightly different way, accounting for the change in ion selectivity.

It is unlikely that all bacterially over-expressed, OG/DTT-solubilised tMOMP is refolding to native conformation. Indeed, it may be bilayer insertion itself that promotes the correct folding of very small amounts of the solubilised proteins. This may also explain the native conformation attained by tMOMP

expressed within the *E. coli* T7 S30 extract system. Although this system is cell-free, it contains a crude extract of ruptured *E. coli* cells which is likely to contain membrane fragments. Spontaneous insertion of recombinant *C. psittaci* MOMP into membrane fragments may help the expressed protein to refold to native. This will be discussed more extensively in Chapter 7.

The ability to reconstitute recombinant MOMP using the lipid bilayer system provides a very sensitive means by which to perform detailed structure/function studies on this important chlamydial antigen. In addition, the possible requirement of membrane insertion in order to achieve native MOMP conformation has broad implications on the design of future sub-unit vaccines.

CHAPTER SIX

RESULTS:

CHLAMYDIA PSITTACI OEA ISOLATE GYCOSAMINOGLYCAN-DEPENDENT INFECTION OF EUKARYOTIC CELLS

6.1 Introduction

The mechanisms by which chlamydiae attach to, and infect, eukaryotic host cells are largely unknown. The initial binding of a bacteria to a target cell is a critical determinant of cell and tissue tropism and thus of pathogenesis. Surface components involved in attachment are likely to be key virulence determinants and primary targets for future vaccine development.

Perhaps the most convincing current hypothesis for chlamydial attachment to eukaryotic cells is that proposed by Zhang and Stephens (1992) (Section 1.4.1). This involves the synthesis of a molecular mimic of heparan-sulphate by *Chlamydia*, which can bind to host cells exploiting receptors used for eukaryotic cell-cell interactions. Evidence supporting this hypothesis includes the inhibition of attachment of radio-labelled *C. trachomatis* (Chen and Stephens, 1997) and fluorescently labelled *C. psittaci* (Gutierrez-Martin *et al.,* 1997) to eukaryotic host cells by the addition of heparin or heparan sulphate and the abolition of infectivity by the treatment of chlamydiae with heparan sulphate lyase. Undoubtedly the most compelling evidence is the restoration of the ability of heparatinase-treated chlamydiae to attach and infect host cells, by the addition of exogenous heparan sulphate.

Chlamydial outer membrane proteins are thought to bind this molecular mimic of heparan sulphate and present it to the host eukaryotic cell GAG receptors. As yet, the identity of the chlamydial heparan-sulphate binding protein is unknown, however, possible candidates include MOMP. Indeed, the specific binding of a recombinant MOMP fusion protein to epithelial cells is inhibited by the addition of heparan sulphate or by the heparitinase treatment of the cells. Furthermore, the attachment of the recombinant MOMP fusion protein to a mutant cell line defective

in heparan sulphate synthesis was markedly reduced (Su *et al.,* 1996). These results strongly suggest that the MOMP is the chlamydial GAG receptor.

The primary objective of the experiments described in the following chapter was to provide some evidence of a direct association between MOMP and heparin or heparan sulphate. In doing so, it was first necessary to demonstrate that the attachment/infection of McCoy cells by the ovine abortion isolate of *C. psittaci* was mediated by a GAG-dependent pathway. Despite some convincing evidence, it has never been suggested that the GAG-mediated pathway is the only mode of attachment and infection exploited by chlamydiae. Indeed, chlamydial infection is believed to be a multifactorial process. However, the characterisation of at least one potential mode of chlamydial attachment and entry into host cells has been an important step.

6.2 Analysis of the GAG-dependent infection of McCoy cells by the OEA subtype of *C. psittaci*

6.2.1 Dose response inhibition of infection by heparin

Recently, it has been demonstrated that *C. trachomatis* trachoma and LGV biovars share the same GAG-dependent mechanism for infection of eukaryotic cells (Chen and Stephens, 1997). These biovars have been shown to compete for the same receptors on HeLa cells and their infectivity has been inhibited by the addition of exogenous heparin/heparan sulphate. In this study attempts were made to show that the infection of McCoy cells by OEA subtype *C. psittaci* was also mediated by a GAG-dependent mechanism.

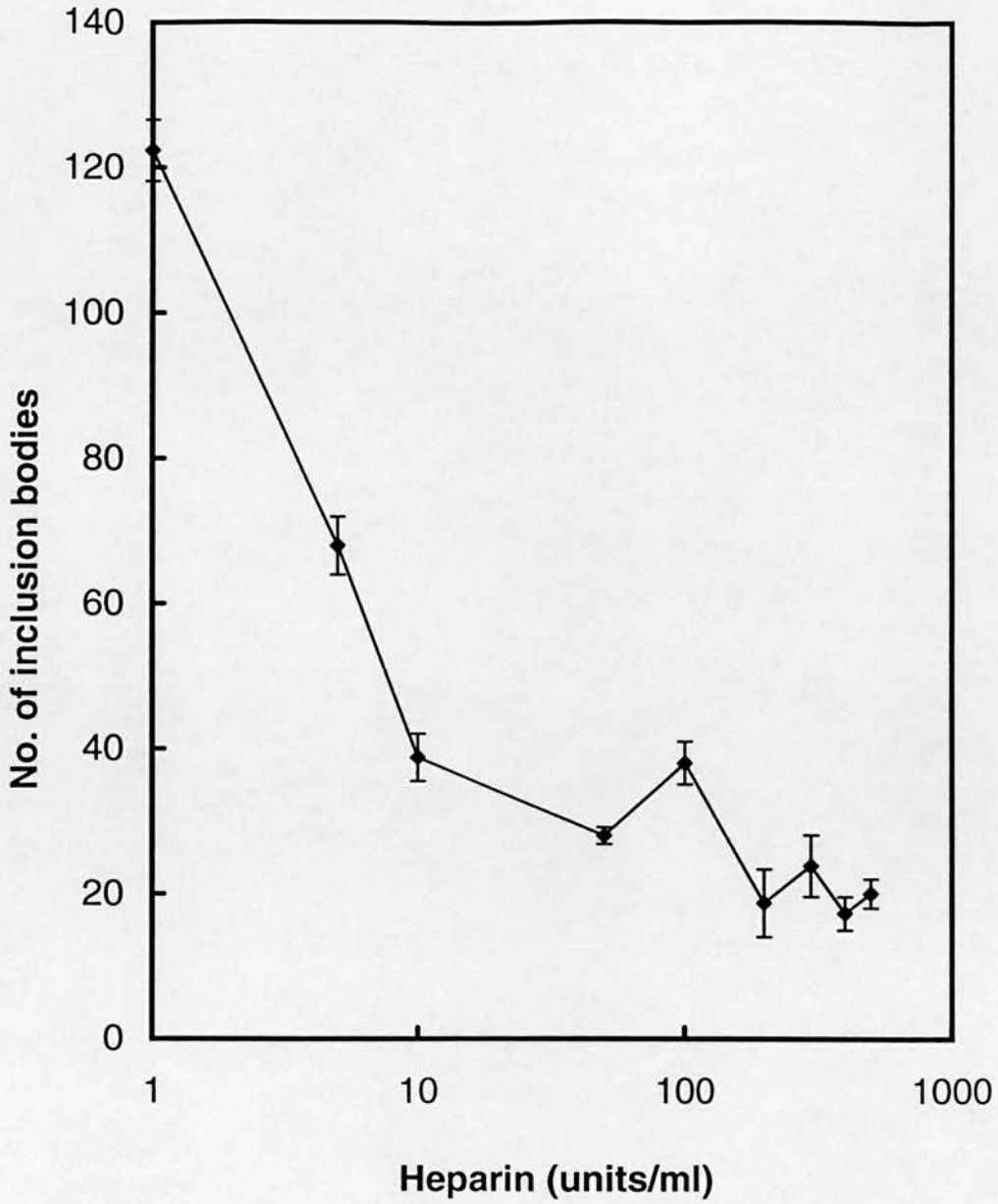


Figure 6.1 Dose-dependent heparin inhibition of *C. psittaci* OEA isolate infection. Error bars represent ± standard error of the mean of triplicate measurements from three separate experiments.

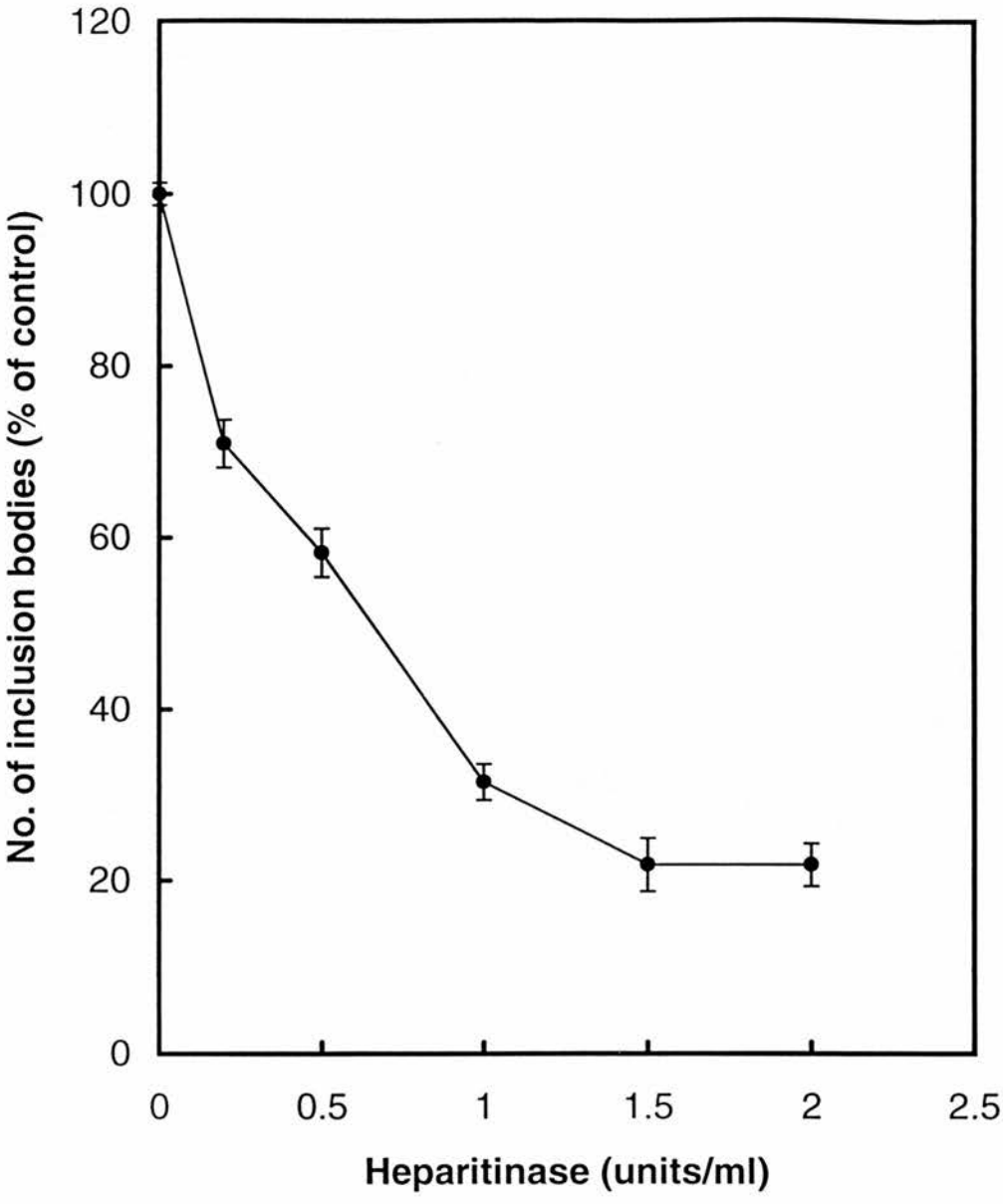


Figure 6.2 Dose-dependent inhibition of *C. psittaci* OEA infection following treatment of chlamydiae with heparitinase. Error bars represent ± standard error of the mean of triplicate measurements from three separate experiments.

The level of *C. psittaci* infection of McCoy cells in the presence of heparin, calculated by a total count of inclusion bodies, exhibited dose-dependent inhibition. As illustrated in Figure 6.1, the level of infection at each concentration of heparin was calculated. Each data point represents the mean of triplicate measurements made from three separate experiments. The addition of 5 units of heparin from ovine intestinal mucosa was sufficient to reduce the infectivity of OEA subtype *C. psittaci* by approximately 44%. Unlike the effect observed using the LGV biovar of *C. trachomatis*, the dose-response curve showed no initial increase in the levels of infection for OEA *C. psittaci* EBs at low levels of heparin. Indeed, infectivity sharply declined upon the titration of increasing amounts of heparin. However, the addition of 100 U/ml of heparin consistently resulted in a small, unexplained increase in the level of infection. Although these results demonstrate that heparin effectively inhibits infectivity of OEA *C. psittaci* this heparin mediated inhibition is not complete. At titrations of heparin as high as 500 U/ml infectivity remained at low levels (16% of the control). This remaining low level of infection may illustrate the adaptability of chlamydiae to use an alternate method of attachment and entry into the host cell. Maximal heparin inhibition of the *C. psittaci* OEA isolate was obtained at approximately 10-50 U/ml of heparin, equivalent to that observed in *C. trachomatis* LGV biovar.

6.2.2 Inhibition of infection by treatment of EBs with heparatinase

Heparitinase cleaves an N-acetyl-D-glucosamine linkage in heparan sulphate. Digestion of LGV biovar EBs with this enzyme resulted in the neutralisation of infectivity and attachment. In this study, the digestion of EBs with heparitinase was attempted to confirm the GAG-dependent mechanism of infection

in the *C. psittaci* OEA isolate. Inhibition of infectivity was found to be dose-dependent following treatment of EBs with heparitinase with maximum inhibition occuring with 1.5 U/ml heparitinase (Fig. 6.2). Furthermore, infection was not totally abolished following digestion treatment with 2 U/ml heparitinase (22% of original infection remained). It is possible that levels of infection could have fallen still further had increasing amounts of heparitinase been analysed. The reduction in chlamydial infectivity observed following the treatment of EBs with heparitinase was consistent with the inhibition of infectivity upon the addition of exogenous heparin.

6.2.3 Association of oligomeric MOMP and [*N* - Sulphonate-^{35}S]Heparin

In view of the results of Su *et al.* (1996) suggesting that MOMP may be the chlamydial GAG-receptor, an attempt was made to show a direct association between oligomeric MOMP and radioactively labelled heparin. MOMP, solubilised in OG-DTT, was run on SDS-PAGE gels under relatively non-denaturing conditions, as described in Section 2.2. Under these conditions, MOMP migrates with an apparent molecular mass of 100 kDa which is interpreted to be an oligomer, presumably a trimer. The oligomeric form of MOMP reacts with monoclonal antibodies raised against intact EBs and recognising only conformational epitopes (Section 3.2.1). Western blots of these oligomers were incubated with [*N*-Sulphonate ^{35}S]heparin, washed with PBST, exposed on x-ray film and developed by auto-radiography. Despite varying the exposure time to film and the method of blocking non-specific binding to the membrane, background radioactivity remained obtrusively high. However, ^{35}S-labelled heparin did associate strongly with the

MOMP oligomer, above background levels of radioactivity. Interestingly, this increased heparin binding was not observed on Western blots of denatured, monomeric MOMP suggesting a specific interaction between heparin and the oligomeric form of MOMP. Until a method of blocking membranes can be found which can reduce the non-specific binding of heparin, the possible specific interaction between oligomeric MOMP and heparin cannot be fully characterised using this technique.

6.3 Discussion

When studying mechanisms of attachment and infection within an *in vitro* system it is essential to maintain an environment as close to that of the natural infection as possible. Undoubtedly, no *in vitro* model will completely mimic the conditions seen *in vivo*, however, if meaningful comparisons are to be drawn with the natural infectious process, every effort must be made to mirror these natural conditions. The present study has been largely based on the work of Chen and Stephens (1997) on the attachment and infectivity of the *C. trachomatis* LGV and trachoma biovars. Throughout the studies of Chen and Stephens, cells were maintained in tissue culture medium containing cycloheximide. This compound acts as an inhibitor of host cell protein synthesis and increases the susceptibility of the cell to infection by chlamydiae. Clearly under these conditions the resulting infection is not natural. EBs which may have been unable to infect an intact, untreated cell may well have been able to establish infection within a cycloheximide-treated epithelial cell. With this in mind, the McCoy cell cultures used in this study of *C. psittaci*, OEA isolate infection were not maintained in medium containing cycloheximide. This adapted system is undoubtedly closer to

the natural environment *in vivo*.

Despite these small alterations in methodology, *C. psittaci* OEA subtype infection of McCoy cells exhibited the same dose-dependent inhibition, in the presence of heparin, as observed by Chen and Stephens with the *C. trachomatis* LGV and trachoma biovars. Maximal inhibition of infection was achieved using equivalent heparin concentrations to those resulting in the maximum inhibition of LGV attachment. However, unlike the effect on LGV, the dose-response curve showed no increase in infectivity of *C. psittaci* EBs at low concentrations of heparin. As in the case of both LGV and trachoma biovars, *C. psittaci* infection was not entirely abolished by the addition of very high concentrations of exogenous heparin, or by the treatment of EBs with heparitinase. These results suggest that both GAG-dependent and independent mechanisms are involved in *C. psittaci* infection. It may be concluded, however, that OEA subtype infection is dominated by GAG-dependent mechanisms, with >80% infectivity being lost upon treatment with heparitinase and the addition of high levels of exogenous heparin.

Tissue culture medium used in the experiments of Chen and Stephens, as well as others, investigating the possible GAG-dependent infection of *Chlamydia*, contained FCS, which is known to contain unidentified heparin chelating molecules (Ohki and Kohashi, 1993). Clearly the levels of heparin required to inhibit mechanisms of attachment and infection in these studies may be a great deal lower in the absence of the heparin-binding FCS. The validity of adding such unphysiological concentrations of heparin to an *in vitro* system and comparing these results with the natural chlamydial infection is surely in question. In the absence of FCS, more physiological concentrations of heparin may be required to produce these inhibitory effects.

In the view of the primary objective of this study, analysis of MOMP as a potential chlamydial GAG receptor, it was not thought necessary to investigate separately the mechanisms of attachment and infection of the OEA isolate of *C. psittaci*. Therefore, from the data presented in this study it cannot be concluded if the *C. psittaci* OEA isolate, like the trachoma biovar of *C. trachomatis,* has a GAG-independent mechanism of attachment while retaining a GAG-dependent mechanism of infection. Alternatively, as in the case of *C. trachomatis* LGV biovar, both *C. psittaci* OEA attachment and infectivity may be GAG-dependent. More in depth analysis is required to answer these remaining questions.

With the discovery that addition of exogenous GAGs inhibited attachment and infection of *C. trachomatis* LGV and trachoma biovars (Chen and Stephens, 1997) and the GPIC strain of *C. psittaci* (Gutierrez-Martin *et al.,* 1997), Stephens proposed a highly plausible and unique model for chlamydial infection of eukaryotic cells. He hypothesised that a heparan sulphate-like ligand, bound to the surface of *Chlamydia* by a chlamydial GAG receptor, mediated infectivity by bridging the bacteria and mammalian cell receptors. As the predominant protein of the outer membrane, it has long been hypothesised that MOMP plays a key role in the mechanisms of attachment and infection of chlamydiae. Naturally, MOMP was the primary candidate for the heparan sulphate-like ligand receptor. Direct evidence supporting this hypothesis was recently provided by Su *et al.* (1996). These researchers discovered that the specific binding of a MOMP fusion protein to epithelial cells was inhibited, in a dose-dependent manner, by the addition of exogenous heparin. These results suggest that MOMP potentially functions as a chlamydial GAG receptor.

To illustrate any specific interaction between MOMP and heparin, Western blots of oligomeric MOMP were probed with ^{35}S-labelled heparin. Unfortunately, the extremely high background levels of radioactivity, resulting from the non-specific binding of heparin to the membrane, obscured any potentially specific interaction between heparin and MOMP. However, ^{35}S-labelled heparin did appear to associate strongly with the MOMP oligomer, above background levels of radioactivity. Increased heparin binding was not observed on Western blots of denatured, monomeric MOMP. Should these results be confirmed, after non-specific binding to membranes has been reduced, they suggest that a specific interaction is taking place between heparin and oligomeric MOMP and that heparin may be recognising a conformational epitope. Addition of increasing amounts of unlabelled heparin to these Western blots, would result in a dose-dependent decrease in the binding of radioactive heparin, should the association between MOMP and heparin be that of receptor-ligand. Hopefully a successful method of blocking these Western blots can be found and this work can be attempted.

The significance of these results depends upon the acceptance that Western blots of MOMP, unboiled in SDS sample buffer, retain some of the structural information of native MOMP. Clearly this is the case, when antibodies raised against intact EBs recognise the oligomeric form of MOMP while failing to recognise the denatured monomer (Section 3.2.1). The recognition of conformational epitopes of native MOMP by radioactively-labelled heparin validates the use of this experimental approach as a simplified model of natural infection.

Previous investigations of binding between GAGs and proteins have indicated the importance of contiguous clusters of basic amino acids within the

protein primary sequence in the binding of heparin, with charge interactions thought to be of primary importance (Cardin and Weintraub, 1989). Analysis of the amino acid sequence of MOMP from *C. psittaci* OEA isolate reveals the presence of a cluster of basic residues just downstream of the surface exposed VS IV region of the protein. This run of basic amino acids (K_K_RK, amino acids 324-329) may form an extended heparin binding site forming part of the surface exposed loop of the VS IV region. Similar motifs have been identified in many organisms, in particular within the envelope proteins of the flaviviridae (Chen *et al.,* 1997).

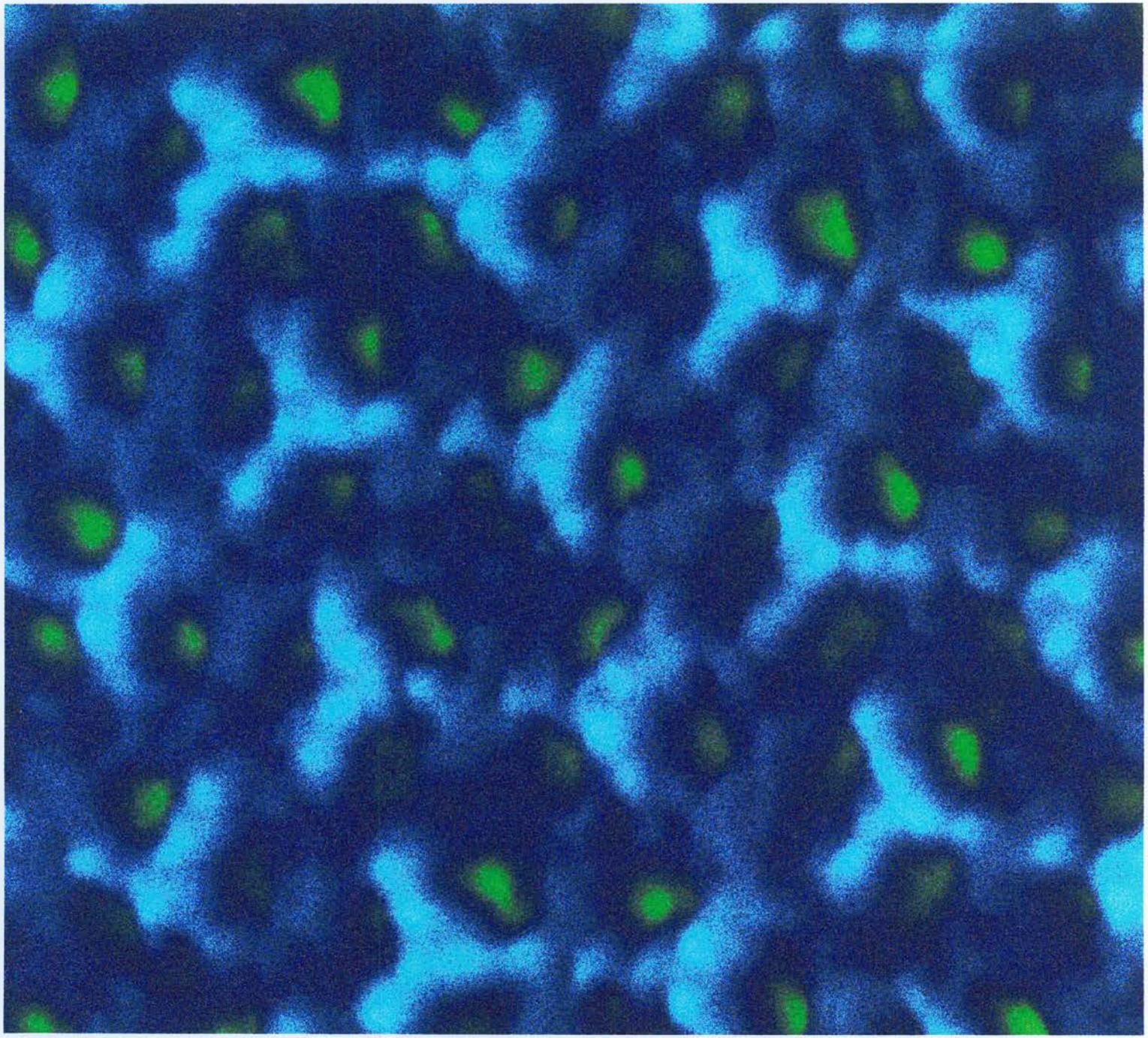
CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE WORK

The initial aims of this study were to characterise structurally and functionally the MOMP of *C. psittaci*. In particular, to investigate fully the MOMP porin hypothesis, at the molecular level, using planar lipid bilayer reconstitution and, at the structural level, by CD analysis of the purified protein. The assignment of a definite function to MOMP, in addition to more detailed structural information, should help to improve the design of future MOMP-based anti-chlamydial vaccines.

Purification of sufficient quantities of purified MOMP for structural and functional analysis has long been hindered by two main factors. Firstly, the obligate intracellular mode of growth of chlamydiae has hampered the large-scale production of native chlamydial proteins for purification. Secondly, it is extremely difficult to solubilise and purify a protein such as MOMP which is both highly disulphide bond cross-linked and normally resides in a hydrophobic environment. Furthermore, in order to get an accurate determination of secondary structure by CD analysis, MOMP was required to be at least 90% pure. The most effective method of purification, which also yielded protein of sufficiently high concentration for CD analysis, was hydroxyapatite chromatography in the presence of SDS. Such analysis showed that MOMP predominantly consists of a β-sheet secondary structure (62%). No α-helix was detected. The high ß-sheet content of MOMP is similar to that calculated for bacterial porins previously characterised by X-ray crystallography (Cowan *et al.,* 1992). The relatively high proportion of random coil (38%), calculated from the MOMP spectrum, may be attributed to its four surface exposed variable segments. A preliminary structural model of MOMP incorporating this information will be discussed more fully later.

OG-DTT-solubilised MOMP reconstituted into planar lipid bilayers incorporated spontaneously into the bilayer giving rise to ion channel-like unit currents. Channel incorporation appeared to be autocatalytic, in that the rate of incorporation accelerated markedly following the initial appearance of channel unit currents. These results suggest that once one MOMP channel has inserted it may act as a catalyst for further incorporation. This hypothesis could be confirmed using the technique employed by Xu and Colombini (1996) in demonstrating the self-catalysed insertion of the VDAC mitochondrial porin. In the study, the addition of urea or guanidinium chloride (GdmCl) to the bilayer following initial channel bilayer incorporation significantly accelerated the already apparent auto-catalytic insertion of VDAC. However, no channel insertion occurred when GdmCl or urea was added without prior incorporation of porin channels emphasising the importance of pre-existing channels to catalyse the membrane insertion process. It was suggested that urea and GdmCl accelerate this process by inducing structural changes in channels already incorporated into the bilayer resulting in these channels becoming better insertion catalysts. The preference shown by MOMP for a particular bilayer orientation was demonstrated by the asymmetric response of the channel to holding potentials of opposite polarity. This functional asymmetry almost certainly has a structural basis which may help explain the process of self-catalysed insertion. A preferred orientation suggests that there is an energetically more favourable bilayer arrangement, presumably due to some MOMP structural element. Once a single channel has spontaneously inserted into the bilayer in this conformation it may interact with channels still in solution orientating them into this low energy conformation and thereby facilitating and accelerating subsequent fusion.



In view of the modification of channel behaviour by MOMP oligomer-specific mAb A11 and subsequent analysis of channel unit currents, a basic model of the functional MOMP porin was developed, where MOMP inserts into the bilayer as a trimer giving rise to 3 pores which traverse the membrane. This hypothesis is consistent with the well characterised homo-oligomeric nature of MOMP previously revealed by biochemical analysis (McCafferty *et al.,* 1995), and the "triple-barrelled" behaviour of other porins (Nakae, *et al.,* 1979). The ultimate confirmation of this proposed "triple-barrelled" MOMP model requires highly sophisticated structural analysis. A long term aim must be to solve a representative chlamydial MOMP crystal structure, possibly by molecular replacement analysis based on *E. coli* OmpF or *R. capsulatus* porin. An alternative technique which often requires no sample preparation, allowing the direct observation of native samples under native or near-native conditions is atomic force microscopy. Using these high resolution microscopes which have three dimensional measurement capabilities, the barrels of individual porin trimers are clearly visible (Fig. 7.1). Although this technique may provide a short term answer to this question, more detailed structural information will still require analysis by x-ray crystallography.

Although mAb A11 affects both the gating and permeation of native MOMP, its epitope, being conformationally-dependent, is unknown. However, it is likely to be a sequence in the outer channel vestibule, which is surface exposed and presumably immunogenic. This is consistent with the idea that the bound antibody obstructs ion movements and intermittently occludes the channel, giving rise to the observed "flickery" closing events. Indeed, these "flickery" closures are reminiscent of the sub-conductance states observed in many MOMP channel recordings (Section

4.2.3, Fig. 4.2). It may be the case that binding of mAb A11 locks the channel in one of these many substates. MAb A11 has been shown to neutralise *C. psittaci* infection *in vitro* (Buzoni-Gatel *et al.,* 1990). It is tempting to speculate that the partial occlusion of the MOMP channel by A11, demonstrated in bilayers, accounts for the neutralisation of infection by preventing nutrient acquisition from the host cell, or perhaps by preventing the acquisition of molecules required to trigger development. This may explain why so many antibodies specific to MOMP neutralise chlamydial infection.

With the identification of genes encoding ATP biosynthetic pathways by the *Chlamydia* Genome Sequencing Project (Stephens *et al.,* 1998) it now seems unlikely that *Chlamydia* derive all their ATP from the host cell, as previously thought. However, the passage of substantial amounts of ATP was demonstrated through the chlamydial porin. It may be the case that host nucleoside triphosphates are required to 'kick start' the metabolic activity of the RB following conversion from the EB in the early stages of the developmental cycle. In truth, ATP may not be the only thing to pass through MOMP porins. As demonstrated by ion selectivity studies, MOMP channels are relatively unselective, particularly in comparison to the *E. coli* porins which tend to be strongly cation selective (Nikaido, 1994). Perhaps the MOMP chlamydial porin provides a means by which chlamydiae can non-selectively 'sample' the contents of the host cell cytoplasm. In other porins, the channel vestibule is an important region in terms of ionic selectivity. Investigation of the amino acid residues of the MOMP channel vestibule by chemical modification and, in the future, by site directed mutagenesis of recombinant MOMP porins, may reveal more about the channels selectivity and physiological role.

Enriched MOMP samples reconstituted in both this study and liposome swelling studies (Bavoil *et al.,* 1984) were solubilised in OG/DTT, however, they may have originated from two different developmental forms of chlamydiae perhaps explaining their different requirement for reducing agent. MOMP reconstituted in liposome swelling studies was recovered from fractions of purified EBs while MOMP used in this study was recovered from a mixture of both EBs and RBs. The extensive disulphide bond crosslinking of the EB outer membrane almost certainly holds MOMP porins in a tightly closed and constricted conformation in order to maintain osmotic stability in an extracellular environment. It may be the case that the addition of reducing agent during purification is not sufficient to relax the tightly compacted conformation of MOMP purified from EB outer membranes, and cysteine residues which are in close proximity in this conformation spontaneously reform disulphide bonds effectively closing the channel. It is clear that MOMP's pore forming activity would be primarily utilised at the RB stage of the chlamydial lifecycle and MOMP purified from RB outer membranes is likely to be in a functionally active, relaxed conformation unlikely to spontaneously reform disulphide bonds resulting in the closure of the porin. It may not be possible to fully investigate the significance of disulphide bond crosslinking in the activation/inactivation of MOMP porins using the native protein. Functional reconstitution of a recombinant MOMP provides a system in which the importance of individual cysteine residues on porin behaviour can be directly assessed by site directed mutagenesis.

The reconstitution of recombinant *C. psittaci* and *C. pneumoniae* MOMPs reported in this study represents the first time native function has been demonstrated for any recombinant chlamydial MOMP. The successful reconstitution of *C.*

pneumoniae MOMP is particularly significant. It has been proposed that, in contrast to the MOMPs of the other chlamydial strains, *C. pneumoniae* MOMP is less immunogenic and antigenically complex (Campbell *et al.,* 1990). Indeed, Christiansen *et al.* (1998) reported that MOMP could not be detected on the surface of *C. pneumoniae* EBs. These researchers hypothesise that the OMP90 family of proteins form a folded layer at the surface of *C. pneumoniae* effectively shielding MOMP from exposure on the surface. In view of the porin-like function of *C. pneumoniae* MOMP demonstrated in this study, this hypothesis seems unlikely. It is difficult to see how any porin can efficiently transport nutrients while lying beneath a protective layer of proteins. A commercially available *C. pneumoniae*-specific mAb and sera raised against the denatured recombinant protein both failed to affect recombinant channel activity. As discussed previously (Section 5.5), there may be some doubt regarding the specificity of this commercial mAb. The sera raised against recombinant *C. pneumoniae* MOMP almost certainly recognises sequence specific linear epitopes. As exemplified by studies on the *C. psittaci* porin, antibodies recognising conformational epitopes are more likely to affect channel characteristics than those specific to linear epitopes which may be buried or inaccessible in the folded protein. Undoubtedly, future studies of the *C. pneumoniae* MOMP channel should include a detailed comparison of the single channel properties of the native and recombinant porin. Ideally, a mAb that recognised conformational epitopes on the native protein and affected native channel function could be used to determine the native or near-native conformation of the recombinant porin. Few candidate mAbs have been reported, however, Persson and Peykani (1998) have recently raised mAbs which are specific to an oligomeric form of *C. pneumoniae* MOMP. It will be interesting to observe any

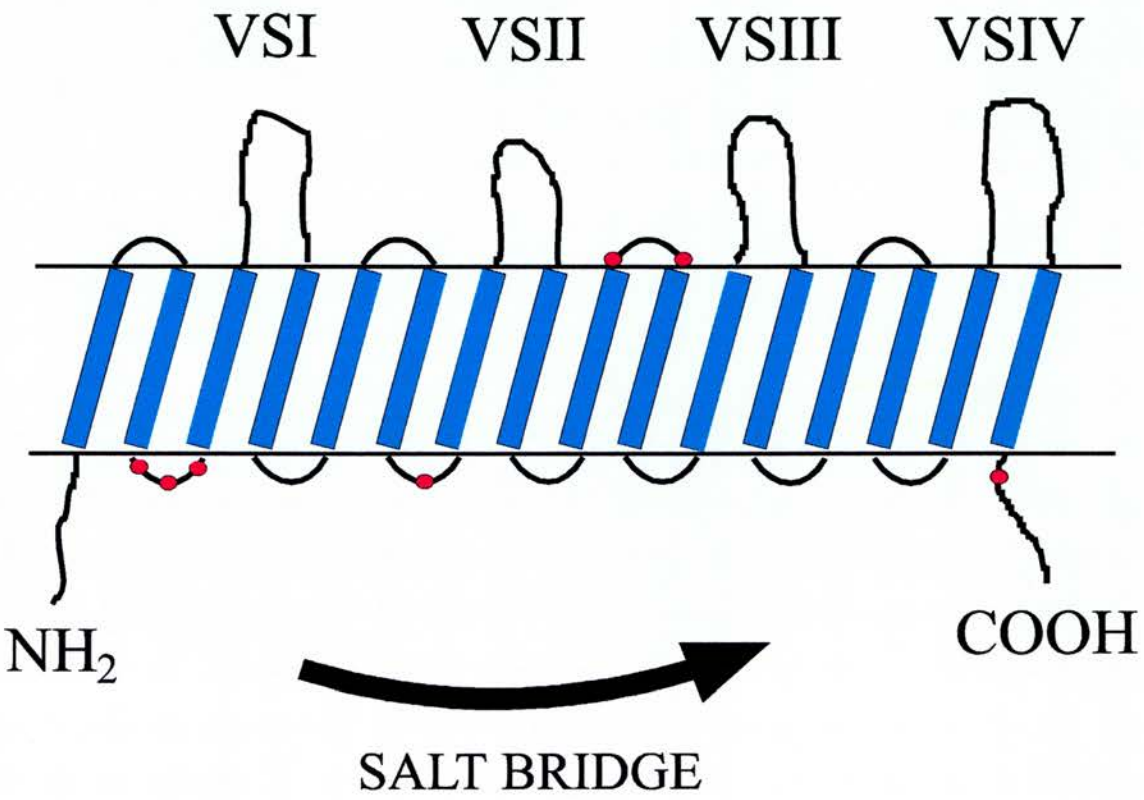


Figure 7.2 Schematic model of *C. psittaci* OEA MOMP transmembrane structure.

Cysteine residues (amino acids 49, 52, 56, 141, 209, 211 and 363).

Transmembrane β-strands (ranging from 8-17 amino acids in length).

Variable regions (VS) VSI (amino acids 85-105), VSII (amino acids 158-178), VSIII (amino acids 238-258) and VSIV (amino acids 305-337).

N-terminal tail 22 amino acids in length and C-terminal tail 35 amino acids in length.

affects these mAbs may have on *C. pneumoniae* MOMP porin function.

Secondary structure information and confirmation of the ion channel function of chlamydial MOMPs firmly places these proteins in the porin channel superfamily. The crystal structures of several porins have been determined and these proteins have been found to form a trimeric β-barrel structure composed of 16 anti-parallel β-strands (Cowan *et al.,* 1992). In view of the results presented in this study, it seems likely that chlamydial porins have a similar structure. The prediction of porin structure from sequence data alone is very difficult, however, an attempt has been made to map the amino acid sequence of OEA *C. psittaci* MOMP to the porin structural model. Information concerning the four surface exposed VSs of MOMP has also been incorporated into this model. As indicated in Figure 7.2, it is possible to form a 16 anti-parallel β-strand structure using the MOMP sequence while maintaining the four surface exposed variable loops. Transmembrane β-strands in this model range from 9 - 17 residues. Interestingly, in this model, many of the conserved cysteine residues, thought to form disulphide bond crosslinks with other envelope and periplasmic proteins, are exposed on the periplasmic surface of the membrane. The amino and carboxy termini of porins are usually linked by a salt bridge within the 16th β-strand of the structure forming a barrel-like structure which is stabilised by inter-strand hydrogen bonds. The channel vestibule is formed by surface exposed loops which extend towards the barrel axis, these loops pack tightly together and are thought to initially limit entry to the pore on the basis of size and charge. It is probable that the four surface-exposed variable loops of MOMP form at least part of the channel mouth and that one of the loops of MOMP acts as the "eyelet", a pore-confined loop that greatly reduces the dimensions of the pore and is

thought to control voltage-dependent gating. There is a strong case for the VS3 region of MOMP being the eyelet. VS3 is the least immunogenic of the four variable regions which may be due to its burial within the pore. In addition, VS3 consists of many charged and polar residues which are characteristic of a porin eyelet. VS2 is also a candidate eyelet region of the MOMP pore. Mab 4/11 recognises a linear epitope within this region of MOMP, however, the addition of 4/11 to the bilayer has no effect on channel properties. The failure of 4/11 to partially occlude the channel suggests that the VS2 loop may be inaccessible to the antibody possibly because it is lining the MOMP pore and controlling voltage-dependent gating. At present we can only speculate as to the identity of the MOMP pore eyelet region. The sequence PEFGG which is conserved within the eyelet region of many members of the porin superfamily (Cowan *et al.,* 1992) is not present within the MOMP sequence. As discussed previously, the crystal structure of a least one chlamydial porin structure is needed before progress is made in determining the important structural elements such as the porin eyelet. In the meantime, site-directed mutagenesis may be used to probe the structure of potential eyelet regions and the loops involved in forming the MOMP channel vestibule.

The reconstitution of recombinant MOMPs to native function and conformation has broad implications on the future design of sub-unit vaccines. As discussed previously (Section 5.1), a successful chlamydial vaccine may need to comprise conformational information in order to elicit a long-lasting, protective immune response. The native function and conformation attained by soluble recombinant MOMP within a membraneous environment suggests a potential means by which these immunogens can be introduced to the immune system inducing protective cellular and humoral immune responses by recalling

conformational native epitopes on the organism. Future vaccine studies could involve the use of liposomes as a vector to present functionally and conformationally native recombinant MOMP. The efficacy of liposomes as vehicles for the presentation of antigens to the immune system has been shown for a number of MOMPs from other bacteria (Gregoriadis, 1990). Liposomes are versatile delivery systems and several factors can be altered to optimise the immune response of liposome-associated antigens. The physico-chemical properties of the liposome itself such as lipid composition, size and charge are particularly important. However, in view of the autocatalytic bilayer insertion of MOMP, reported in various lipid conditions, altering these properties should not unduly effect the presentation of this antigen. In contrast to other delivery mechanisms, liposome-associated antigens do not induce granulomas at the site of injection and hypersensitivity is generally not a problem. The versatility of the liposome delivery system creates a wide range of options for the design of an effective MOMP subunit vaccine. Indeed, the planar lipid bilayer is on hand to assess the native structure and function of these liposomal antigen formulations.

At what point and by what mechanism recombinant MOMP attains native conformation are critical questions which must be addressed. In this study, MOMP, expressed as an insoluble inclusion body within *E. coli*, was incubated in OG/DTT for 1h at 37°C, the insoluble product pelleted and the supernatant reconstituted into bilayers. The conformational status of this OG/DTT solubilised MOMP is unknown. There are two possible scenarios, the small quantities of MOMP solubilised in OG/DTT from insoluble inclusion bodies may contain little or no native conformational information only refolding to native structure upon insertion into the bilayer. Alternatively, small quantities of soluble recombinant MOMP,

already folded into native conformation, may be expressed in association with the inclusion body. This "conformational MOMP", presumably recovered in the supernatant following solubilisation of the inclusion body in OG/DTT, may insert directly into the bilayer. It is crucially important to find out which of these two possibilities is actually taking place. The application of liposome-reconstituted MOMP as a potential vaccine can only work if reasonable amounts of protein can be reconstituted into the bilayer and attain native conformation. If insertion into a membrane environment is sufficient to refold recombinant MOMP to native conformation then inclusion bodies can be solubilised, perhaps using urea, then successfully reconstituted into liposome. However, if a native or near-native conformation is needed for successful membrane insertion it is difficult to see how enough conformational protein can be produced from inclusion bodies, which are usually solubilised by chaotropic agents, to manufacture a viable vaccine. The best way to determine if conformation is required for membrane insertion may indeed be to solubilise the MOMP inclusion bodies using a chaotropic agent such as GdmCl and attempt reconstitution into bilayers.

The initial events of chlamydial pathogenesis, attachment of the infectious EB to a susceptible cell followed by internalisation, remain poorly understood. However, evidence suggesting that a GAG plays a significant role in this process is mounting (Chen and Stephens, 1997; Gutierrez-Martin *et al.,* 1997). Results presented in this study indicate that *C. psittaci* OEA isolate infection is a predominantly GAG-dependent process mirroring the results obtained with the trachoma and LGV biovars of *C. trachomatis* and the GPIC strain of *C. psittaci.*

The chlamydial outer membrane protein which acts as the GAG receptor is unknown, however, due to its predominance in the outer membrane, MOMP remains

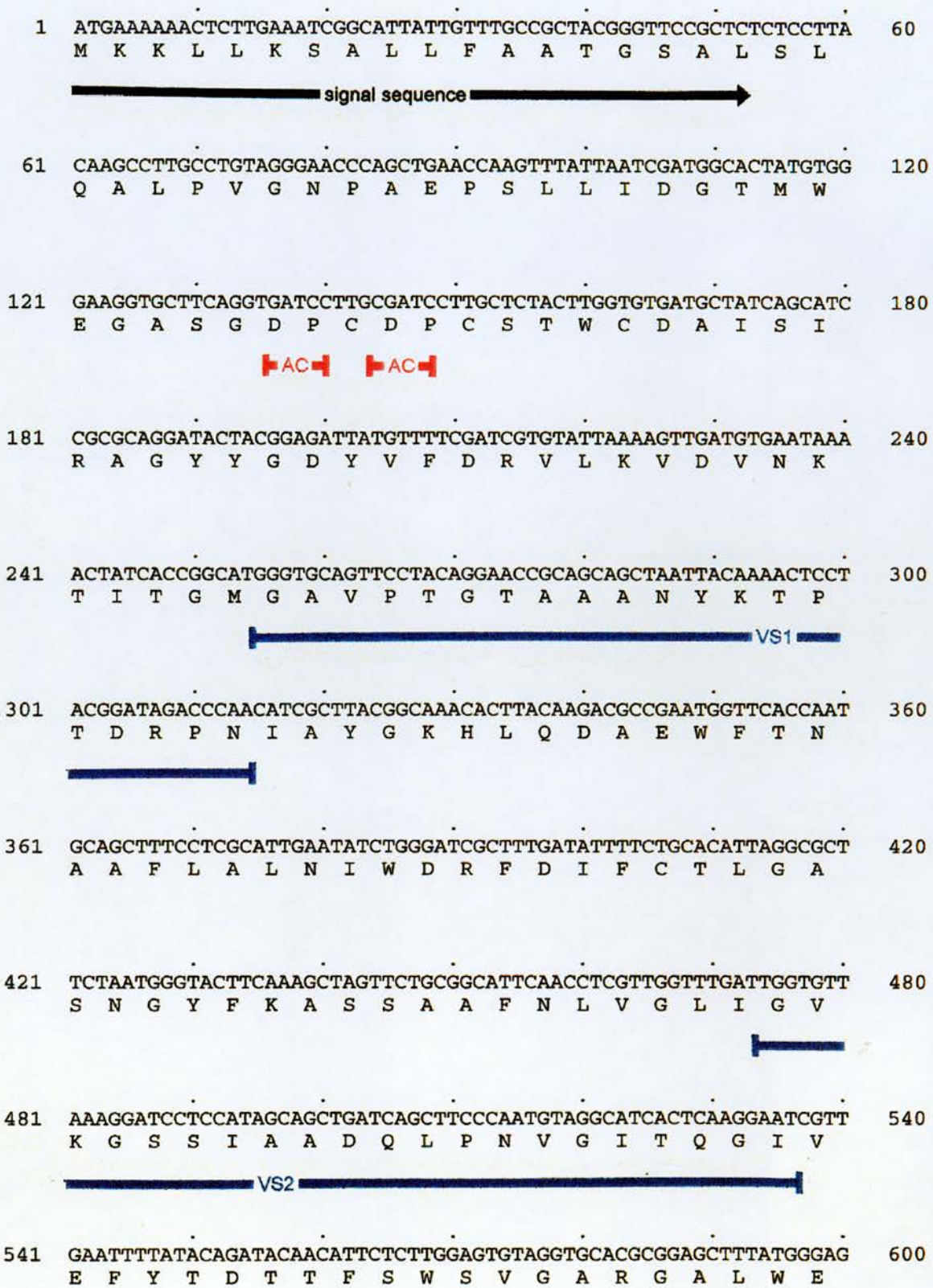
a strong candidate. Interestingly, in close proximity to the surface exposed VSIV region of *C. psittaci* OEA MOMP is a run of basic amino acids (residues 324-329, Appendix I) which is similar to heparin binding motifs identified in many other organisms (Chen *et al.,* 1997). High level non-specific binding of ^{35}S-labelled heparin obscured any potentially specific interaction between the GAG and native MOMP on Western blots. However, labelled heparin did appear to associate strongly with the oligomeric MOMP, above background levels of radioactivity. Clearly, in view of the high non-specific binding of heparin in these experiments, an alternative method of characterising any specific interaction between heparin and conformational MOMP must be found. As reported in Section 3.2.5, the pellet resulting from centrifugation of OG/DTT-solubilised MOMP through a 5-20% (w/v) linear gradient of sucrose was highly enriched for MOMP (85% as estimated by densitometry), therefore an interesting way of investigating the binding of heparin may be to use sucrose density gradients. If OG/DTT-solubilised MOMP was incubated with labelled heparin, and a specific interaction did take place, the label would be expected to co-sediment with MOMP in the pellet following centrifugation. An interesting aspect of this experiment is that other surface exposed outer membrane proteins, such as the members of the OMP90 family, which may also be involved in the process of attachment will be banded on this gradient and any specific interaction between heparin and these proteins can also be characterised.

In the past MOMP has been implicated in almost every aspect of chlamydial pathogenesis. This is perhaps not surprising, considering that most *Chlamydia* research has centred around this fascinating protein. It is clear from the results presented in this study that the primary function of MOMP is as a chlamydial porin. In the future, bilayer reconstitution clearly offers an important route to detailed

structure/function studies on recombinant chlamydial MOMPs and this work also has broad implications for the production of improved immunogenic MOMP protein as future subunit vaccines.

APPENDIX

Appendix I The DNA and amino acid sequence of *C. psittaci* OEA MOMP. The four variable regions of the amino acid sequence are labelled (VS1-4). Regions of the protein sequence susceptible to cleavage in mild acidic conditions are labelled (AC) while a putative heparin binding site (PHBS) is also denoted.


1 ATGAAAAAACTCTTGAAATCGGCATTATTGTTTGCCGCTACGGGTTCCGCTCTCTCCTTA 60
M K K L L K S A L L F A A T G S A L S L
signal sequence
61 CAAGCCTTGCCTGTAGGGAACCCAGCTGAACCAAGTTTATTAATCGATGGCACTATGTGG 120
Q A L P V G N P A E P S L L I D G T M W
121 GAAGGTGCTTCAGGTGATCCTTGCGATCCTTGCTCTACTTGGTGTGATGCTATCAGCATC 180
E G A S G D P C D P C S T W C D A I S I
AC
AC
181 CGCGCAGGATACTACGGAGATTATGTTTTCGATCGTGTATTAAAAGTTGATGTGAATAAA 240
R A G Y Y G D Y V F D R V L K V D V N K
241 ACTATCACCGGCATGGGTGCAGTTCCTACAGGAACCGCAGCAGCTAATTACAAAACTCCT 300
T I T G M G A V P T G T A A A N Y K T P
VS1
301 ACGGATAGACCCAACATCGCTTACGGCAAACACTTACAAGACGCCGAATGGTTCACCAAT 360
T D R P N I A Y G K H L Q D A E W F T N
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A A F L A L N I W D R F D I F C T L G A
421 TCTAATGGGTACTTCAAAGCTAGTTCTGCGGCATTCAACCTCGTTGGTTTGATTGGTGTT 480
S N G Y F K A S S A A F N L V G L I G V
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K G S S I A A D Q L P N V G I T Q G I V
VS2
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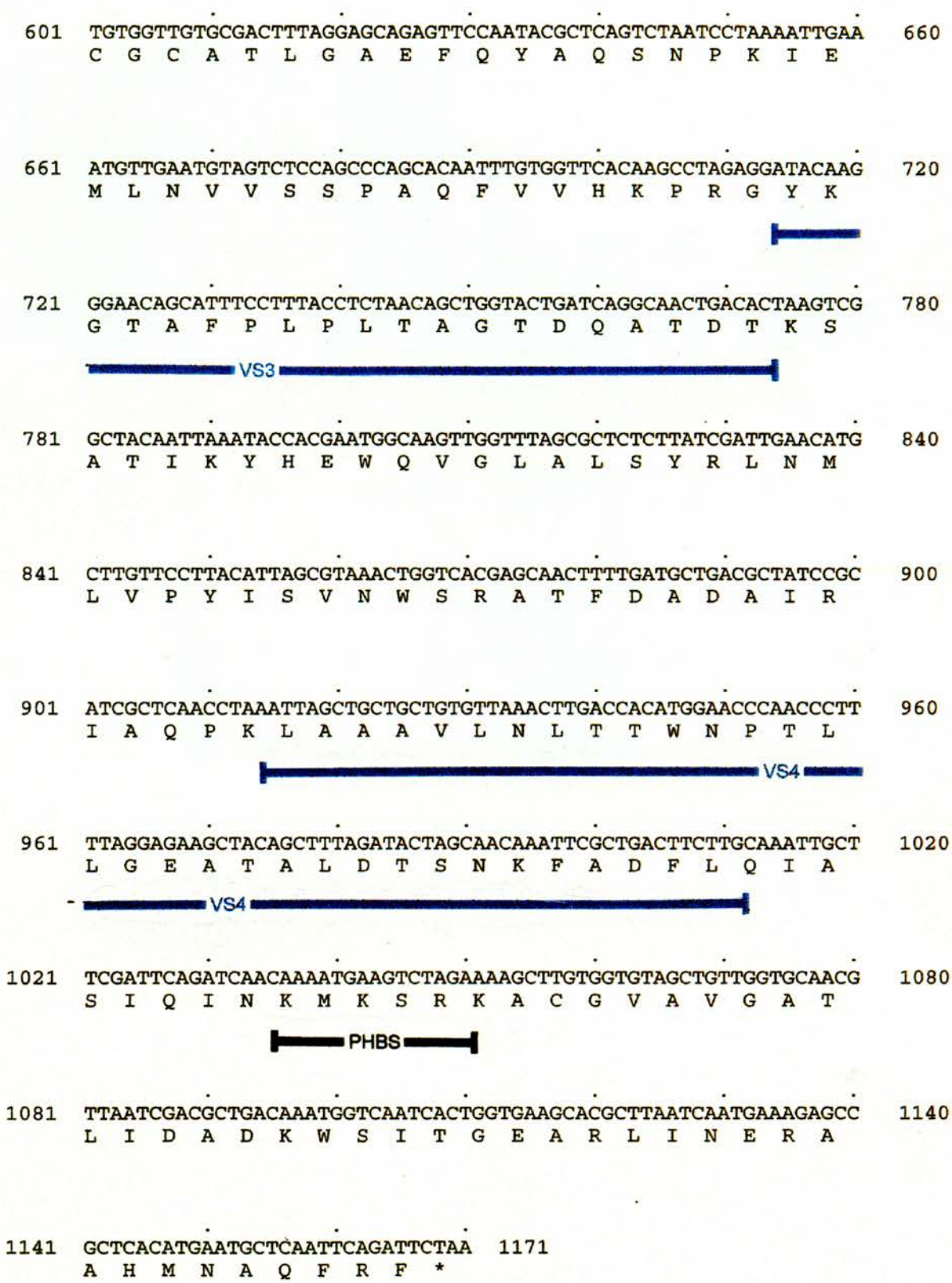
E F Y T D T T F S W S V G A R G A L W E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M L N V V S S P A Q F V V H K P R G Y K
721 GGAACAGCATTTCCTTTACCTCTAACAGCTGGTACTGATCAGGCAACTGACACTAAGTCG 780
G T A F P L P L T A G T D Q A T D T K S
VS3
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A T I K Y H E W Q V G L A L S Y R L N M
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901 ATCGCTCAACCTAAATTAGCTGCTGCTGTGTTAAACTTGACCACATGGAACCCAACCCTT 960
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VS4
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VS4
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PHBS
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1141 GCTCACATGAATGCTCAATTCAGATTCTAA 1171
A H M N A Q F R F *

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The Major Outer Membrane Protein of *Chlamydia psittaci* Functions as a Porin-Like Ion Channel

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The major outer membrane protein (MOMP) of *Chlamydia* species shares several biochemical properties with classical porin proteins. Secondary structure analysis by circular dichroism now reveals that MOMP purified from *Chlamydia psittaci* has a predominantly β-sheet content (62%), which is also typical of bacterial porins. Can MOMP form functional ion channels? To directly test the "porin channel" hypothesis at the molecular level, the MOMP was reconstituted into planar lipid bilayers, where it gave rise to multibarreled channels, probably trimers, which were modified by an anti-MOMP monoclonal antibody. These observations are consistent with the well-characterized homo-oligomeric nature of MOMP previously revealed by biochemical analysis and with the triple-barreled behavior of other porins. MOMP channels were weakly anion selective ($P_{Cl}/P_K \sim 2$) and permeable to ATP. They may therefore be a route by which *Chlamydia* can take advantage of host nucleoside triphosphates and explain why some anti-MOMP antibodies neutralize infection. These findings have broad implications on the search for an effective chlamydial vaccine to control the significant human and animal diseases caused by these organisms.

Members of the order *Chlamydiales* are distinguishable from other bacteria by their obligate intracellular mode of growth and their distinctive biphasic life cycle in which the small spore-like extracellular and infectious form, the elementary body (EB), alternates with the intracellular vegetative form, the reticulate body (RB). The four main species currently recognized, *Chlamydia trachomatis*, *C. psittaci*, *C. pneumoniae*, and *C. pecorum*, are diverse pathogens that cause a range of disease in both humans and animals. A common component of all these species is the 40-kDa major outer membrane protein (MOMP), present in both the EB and RB forms. The MOMP is a multifunctional protein which is thought to have a role both in the infectious process (3, 34–36) and in the maintenance of structural rigidity via disulfide bond cross-linking within the EB outer membrane (13, 15, 26).

The antigenic properties of MOMP have been studied in detail since the landmark discovery that MOMP purified from sodium dodecyl sulfate (SDS)-gels was capable of raising antibodies which could neutralize the infectivity of *C. trachomatis* in vitro (6). Protein sequence comparisons of MOMPs both within (33) and between (18) species, combined with epitope mapping studies (8, 43), have shown that the epitopes responsible for neutralization lie within four variable segments. Vaccine preparations based on chlamydial outer membrane complexes, which are highly enriched for the MOMP in its native form, have been shown to be protective against chlamydial disease in sheep (37), guinea pigs (2), and mice (10, 28). However, experimental vaccines based on denatured or nonnative recombinant MOMP preparations have yielded, at best, only partial protection (28). Most recently, protection was demonstrated in mice administered a DNA vaccine comprising only the MOMP gene (42).

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These results clearly make MOMP the primary candidate for a subunit vaccine against chlamydial infection, but despite many years of intensive study, the paucity of structural information leaves unanswered many questions as to how MOMP fulfills its diverse functions. Structural studies are hampered first by the difficulty of growing chlamydiae in bulk and subsequently by problems with purifying and solubilizing a protein which both is highly cross-linked and normally resides in a hydrophobic environment. These factors have precluded attempts to crystallize the protein and have made it necessary to rely on analysis techniques that require relatively small quantities of protein.

A recent report showed that MOMP solubilized with octyl glucoside (OG) in the presence of dithiothreitol (DTT) was oligomeric, with electrophoretic and sedimentation properties consistent with a trimeric structure (21). These oligomers resisted denaturation with SDS in a way similar to that for classical gram-negative porin molecules, which are also trimers (27). The result was consistent with an early observation by Bavoil et al. (3), who used liposome swelling to demonstrate that the chlamydial outer membrane contained pores and, due to its predominance in the outer membrane, that MOMP was the likely pore-forming protein. In this paper, we report direct evidence for porin function obtained by using native, oligomeric MOMP incorporated into planar lipid bilayers. Moreover, due to the traditional view that chlamydiae are required to scavenge ATP from the host cell, we have investigated the transport of ATP through the MOMP channel.

MATERIALS AND METHODS

Chlamydial culture. The ovine abortion isolate of *C. psittaci*, S26/3, was grown in McCoy cells as previously described (22). Briefly, infected cells were grown in RPMI 1640 (Gibco) supplemented with 5% (vol/vol) newborn calf serum, 0.2% (wt/vol) sodium hydrogen carbonate, 1% (wt/vol) HEPES, streptomycin (0.1 mg/ml), nystatin (25 U/ml), gentamicin (5 μg/ml), and cycloheximide (1 μg/ml).

Purification of MOMP. The MOMP used for reconstitution into planar lipid bilayers was purified from McCoy cells as described previously (3, 21). Secondary structural analysis by circular dichroism (CD) was performed on MOMP purified by nondenaturing hydroxyapatite chromatography as previously described (5). Briefly, 15 to 20 mg of EBs/RBs from tissue culture harvests were suspended in 2% (wt/vol) Sarkosyl and incubated for 1 h at 37°C. After centrifugation for 30

min at 100,000 × *g*, the pellet was resuspended in 2% (wt/vol) SDS in phosphate-buffered saline (pH 7.4) and incubated for 1 h at 37°C. Seven milligrams of the MOMP-enriched SDS extract was equilibrated with 0.01 M sodium phosphate (pH 6.4) containing 1 mM DTT and 0.1% (wt/vol) SDS (column equilibration buffer). This material was fractionated by hydroxyapatite chromatography in the continued presence of SDS, using the technique of Moss and Rosenblum (24). Briefly, the MOMP-enriched sample was applied to a preequilibrated hydroxyapatite column (Bio-Rad) which was then washed with equilibration buffer, and samples were eluted with a linear gradient of 0.1 to 0.6 M sodium phosphate (pH 6.4) containing 1 mM DTT and 0.1% (wt/vol) SDS. The fractions showing increased absorbance at 280 nm were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and by immunoblotting using the MOMP-specific monoclonal antibody (MAb) 4/11.

The MAbs used in this study, specific to the monomeric (4/11) and oligomeric (4/11 and A11) forms of MOMP, have been previously described (4, 21). SDS-PAGE and Western blotting were carried out as described previously (21). Protein concentrations were determined by gel densitometry using a Bio-Rad GS-670 imaging densitometer and/or bicinchoninic acid protein assay reagent (Pierce).

CD. CD analysis of hydroxyapatite-purified MOMP was performed on a Jasco J-600 spectrophotometer with a path length of 0.02 cm, averaging 16 scans between wavelengths of 190 nm and 260 nm for each sample. The spectrophotometer was blanked with 0.3 M sodium phosphate (pH 6.4) containing 1 mM DTT and 0.1% (wt/vol) SDS. Secondary structure estimations were obtained by the CONTIN procedure of Provencher and Glockner (30), which essentially compares the spectrum to a database of spectra from known structures.

Planar lipid bilayer reconstitution and channel analysis. MOMP was incorporated into 0.3-mm-diameter planar lipid bilayers cast at room temperature from a 30-mg/ml decane suspension of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, Ala.), using equipment and techniques similar to those described by Williams (38) as used in previous studies (7, 15). The *cis* side of the bilayer was voltage clamped relative to the *trans* side, using a Biologic RK-300 patch-clamp amplifier (Intracel, Royston, United Kingdom). The relative potential applied across the bilayer is called the holding potential or voltage-clamp potential. Transmembrane currents were low-pass filtered (10-kHz cutoff, 8-pole Bessel filter) and digitally recorded. All bilayers used had a conductance of <10 pS and a capacitance of >250 pS. Bilayers of this size have a relatively large capacitance. When large changes are made to the holding potential (e.g., by switching from 0 to ±80 mV), the bilayer momentarily becomes charged, and the charge then dissipates almost immediately to give rise to an exponentially decaying current transient. This is referred to as the bilayer capacitative current transient and is superimposed on channel recordings made immediately after large changes in the holding potential, giving them a characteristic curved appearance.

To incorporate channels, purified detergent-solubilized MOMP was added to the *cis* side to a final concentration of 1 ng/ml in the presence of a salt gradient, 250 mM KCl *cis* versus 50 mM KCl *trans* (buffered with 10 mM Tris-HCl [pH 7.4]). The solutions bathing the bilayer were changed by perfusion (at least 10 volumes) as required. Opening and closing of the ion channels give rise to square-shaped pulses of current which are termed unit currents. Unit currents and holding (voltage clamp) potentials are displayed according to a standard convention, quoting the holding (clamp) potential in the *cis* chamber, with positive transmembrane (upgoing) currents representing a net flux of cations flowing *cis* to *trans*, or a net flux of anions flowing in the opposite direction. Channel recordings were postfiltered (see figure legends) to reduce high-frequency noise and analyzed with the program pClamp 6 (Axon Instruments, Foster City, Calif.).

In the presence of a salt gradient either side of the bilayer, the reversal potential is defined as the holding potential that exactly balances the tendency for ions to diffuse down their chemical concentration gradient. Relative ionic permeabilities were calculated from measured reversal potentials by using appropriate forms of the Goldman-Hodgkin-Katz voltage equation (7, 17). Briefly, with the same monovalent salt in both chambers, $Er = -RT/zF.\ln\{P_C[C]_t + P_A[A]_t/P_C[C]_c + P_A[A]_t\}$, where Er is the reversal potential, P is permeability, A and C are the anion and cation, respectively, and c and t represent *cis* and *trans*, respectively. R is the gas constant (8.314 JK^{-1} mol^{-1}), T is room temperature (298 K), z is valency, and F is the Faraday constant (9.6×10^4 C mol^{-1}). Concentrations were corrected for ionic activities by reference to activity coefficients obtained from standard tables.

Addition of components to bilayer solutions. (i) MAb A11. The MOMP oligomer-specific MAb A11 (final dilution of 1/1,000), which has previously been described (4), was added to both the *cis* and *trans* chambers in 50 mM KCl–10 mM Tris-HCl (pH 7.4)–1 mg of bovine serum albumin (BSA) per ml.

(ii) Oxidizing agents. Ten to 500 mM Cu^{2+}-phenanthroline, hydrogen peroxide, or oxidized glutathione in 50 mM KCl–10 mM Tris-HCl (pH 7.4) was added to the *cis* and *trans* chambers. Channel activity in bilayers bathed in these buffers was monitored over a period of at least 30 min.

(iii) ATP. ATP was added by perfusion to the bilayer in the form of 10 mM Na^+-ATP–10 mM Tris-HCl (pH 7.4) in both the *cis* and *trans* chambers. More highly concentrated solutions were achieved by the addition of Na^+-ATP to the chambers from a concentrated stock solution. For the multivalent ATP^{4-} ion in the presence of Na^+, the theoretical reverse potential for an ATP^{4-}-selective channel was estimated from the Nernst equation. The activity ratio of 100 mM Na-ATP to 10 mM Na-ATP was measured by using the Na^+ ionophore gramicidin (11).

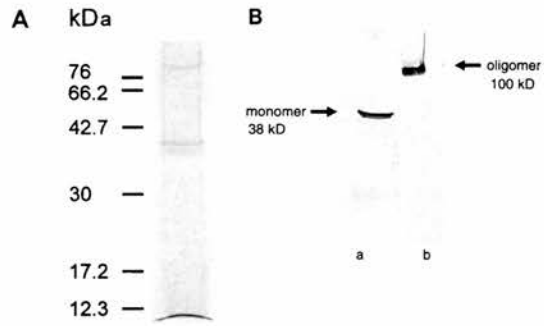

FIG. 1. SDS-PAGE and Western blot analyses of OG-DTT-solubilized MOMP. (A) SDS-PAGE analysis of 2% (wt/vol) OG–10 mM DTT-solubilized MOMP-enriched preparations by silver staining. (B) The OG-DTT-solubilized preparation was subjected to SDS-PAGE on 12.5% gels, immunoblotted, and probed with MAb 4/11 (sample loaded after boiling; lane a) and MAb A11 (sample loaded without boiling; lane b). Molecular masses are indicated.

RESULTS

SDS-PAGE and immunoblotting analysis of OG-DTT-solubilized MOMP. The results of SDS-PAGE and immunoblotting analyses of the MOMP-enriched fraction solubilized with OG-DTT (Fig. 1) illustrate the purity of the MOMP preparation used in planar lipid bilayer reconstitution. Silver staining identified a 38-kDa protein corresponding to the MOMP; contaminant proteins of approximately 90 kDa were also observed (Fig. 1A). These contaminants were identified as the previously described OMP90 (formerly POMP) family (19, 20) by immunoblotting using OMP90-specific MAbs (results not shown). Western blots probed with MAbs specific for the oligomeric (Fig. 1B, lane b) and monomeric (Fig. 1B, lane a) forms of MOMP demonstrated that when MOMP is solubilized in SDS sample buffer at room temperature in the presence of a reducing agent, it migrates with an apparent molecular mass of 100 kDa, which we interpret to represent an oligomer. When fully denatured by boiling, MOMP migrates as a monomer of 38 kDa. These results are typical of those observed with gram-negative porins and are identical to those previously shown by McCafferty et al. (21).

Secondary structure determination. Figure 2 shows silver-stained SDS-PAGE analysis of fractions eluted from a hydroxyapatite column under relatively nondenaturing conditions. Although fractions 20 to 30 contained significant amounts of MOMP oligomer (100 kDa), only fractions 25 to 30 contained very few contaminant proteins while retaining significant amounts of oligomer. MOMP oligomer accounted for >90% of the hydroxyapatite-purified protein (as estimated by densitometry), achieving the criteria for accurate structural analysis by CD. Fractions 25 to 30, which were eluted at a sodium phosphate concentration of approximately 0.3 M, were subjected to CD analysis in the far-UV range; the spectrum obtained for fraction 25 is shown in Fig. 3. The spectrum of purified protein is consistent with the presence of a large percentage of β structure, as indicated by the characteristic absorption minima at approximately 215 nm. Analysis using the CONTIN procedure (30) (see Materials and Methods) estimated 62% β structure, 38% random coil, and 0% α helix. The CD spectrum of bovine heart mitochondrial porin in 0.1% (wt/vol) OG, recorded on the same spectrophotometer and known to contain 60% β structure (29), is shown for comparison.

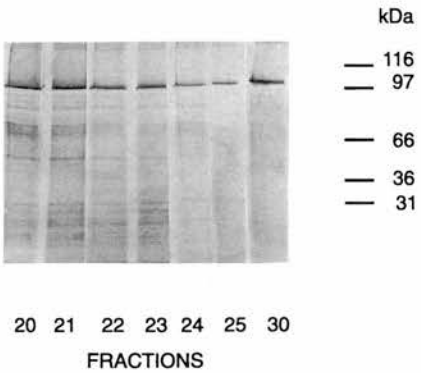

FIG. 2. SDS-PAGE analysis of the hydroxyapatite-purified MOMP-enriched preparation. Fractions eluted from the hydroxyapatite column (5 by 1 cm) loaded with the 2% (wt/vol) SDS-solubilized MOMP-enriched preparation (see Materials and Methods) were subjected to SDS-PAGE on a 12.5% gel, under reducing conditions (not boiled), and silver stained. Molecular masses are indicated.

Planar lipid bilayer reconstitution. OG-DTT-solubilized MOMP incorporated into planar lipid bilayers within 5 to 10 min of addition, to give rise to ion-channel-like unit currents (see Materials and Methods). Unit currents were not seen at 0 mV, despite the presence of an ion gradient (250 versus 50 mM KCl [see Materials and Methods]). This finding suggested that channel opening, or insertion, was a voltage-dependent process. Channel incorporation appeared to be autocatalytic, because the rate of incorporation accelerated markedly following the initial appearance of unit currents (40). After the first evidence of channel incorporation, the solution in the *cis* chamber was changed to limit the channel content of the bilayer. Finally, it was confirmed that the addition of buffer alone had no effect.

Figure 4 shows typical traces, with the same solutions on both the *cis* and *trans* side of a bilayer that had been exposed to MOMP. The data were obtained by switching the holding potential from 0 to either +80 or −80 mV as indicated. Bursts of channel openings are superimposed on bilayer capacitative current transients (see Materials and Methods). The channels exhibit voltage-dependent closure as the high holding potentials are maintained. Normally, the appearances on switching were similar to those shown in Fig. 4, with more open/closed transitions at negative compared to positive holding potentials, but in some experiments all channels became incorporated in the opposite orientation. In addition, the bursts of activity seen after switching to negative holding potentials normally contained more unit currents than those seen at positive potentials. In the presence of diphytanoyl phosphatidylcholine (choline because it is a very robust bilayer lipid), the membrane potential could be clamped as high as ±200 mV without breakage, but the maximum number of unit currents generally appeared to occur at ±60 mV or above.

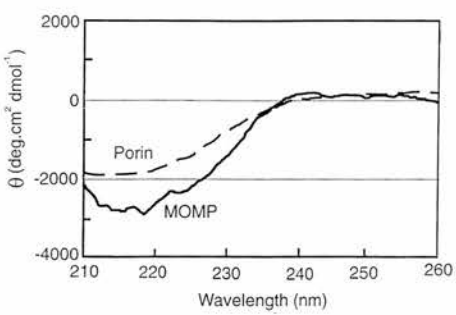

FIG. 3. CD spectra of hydroxyapatite-purified MOMP and bovine heart mitochondrial porin in the presence of 0.1% (wt/vol) OG.

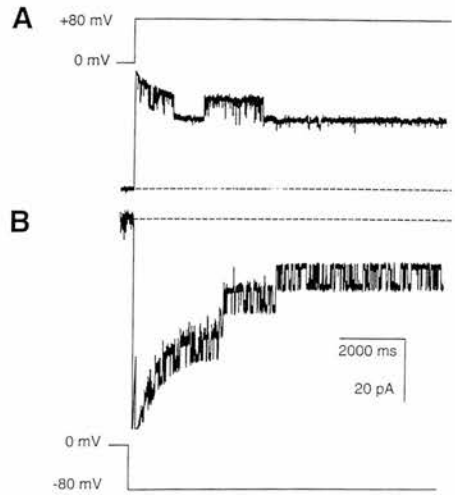

FIG. 4. Ion channel recordings from OG-DTT-solubilized MOMP in a bilayer bathed in 50 mM KCl–10 mM Tris-HCl (pH 7.4). (A) Holding potential switched from 0 to +80 mV. (B) Holding potential switched from 0 to −80 mV. Recordings were low-pass filtered at 100 Hz and show decaying capacitative transients representing the discharge of the typically large bilayer capacitance (~300 pF).

Two types of experiment confirmed that MOMP was the channel-forming protein. First, channel behavior (and rate of channel incorporation) obtained by using similar quantities of MOMP that had been highly purified by hydroxyapatite chromatography was identical to that seen with OG-DTT-solubilized MOMP. This fraction was not reconstituted routinely, because its SDS content destabilized the bilayer. Second, addition of the native MOMP-specific MAb A11 affected both the open/closed transitions of the channel and the amplitude of the unit currents (Fig. 5). These effects could not be reversed by perfusion with antibody-free solutions. Note that the channels did not close completely in the presence of A11 (Fig. 5B). Additions of other immunoglobulins or BSA (1 mg/ml) were without effect (results not shown).

Properties of unit MOMP channels. Given that native MOMP tends to migrate as an oligomer, presumably a trimer, under nondenaturing conditions on SDS-PAGE, and knowing that many classical porins are trimers (9, 23, 25), we measured the number of functional unit currents that could be obtained following bilayer reconstitution of MOMP. The number of individual unit currents (e.g., levels 1, 2, and 3 in Fig. 6A) were counted in 25 independent experiments in which incorporated channels were maximally activated by switching between +120 and −120 mV. The numbers of discrete conducting units are summarized in Fig. 6B.

Unit currents were measured (pClamp 6) to construct open-channel current-voltage relationships in symmetrical KCl (the same concentration of KCl in both the *cis* and *trans* chambers) (Fig. 7). These were all linear; the slope conductances were 120 ± 18, 210 ± 25, and 310 ± 32 pS in 50, 150, and 300 mM KCl, respectively (mean ± standard deviation [SD], $n = 4$). Careful inspection of the data revealed that channel closure was incomplete, with a residual conductance equivalent to approximately 5% of the fully open state. In some recordings we also observed subconductance states, but these substates,

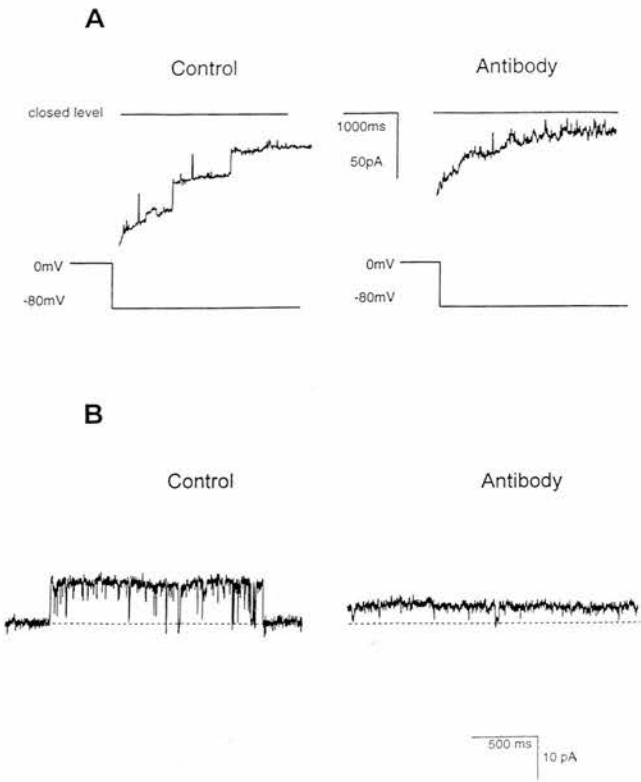

FIG. 5. Effects of MAb A11 on the MOMP channel. The bilayer was bathed in 50 mM KCl–10 mM Tris-HCl (pH 7.4); MAb A11 was added to both the *cis* and *trans* chambers to a final dilution of 1/1,000 in the presence of BSA (1 mg/ml). (A) Traces obtained after switching the holding potential from 0 to −80 mV before (left) and 30 min after (right) addition of MAb A11. In each recording, there is a typical bilayer capacitative current transient superimposed on channel openings, but the openings are of much lower amplitude after addition of MAb A11. (B) Detailed appearance of an individual unit current before (left) and 30 min after (right) addition of MAb A11. Note the difference in amplitude and the reduction in the number of long-lived channel closures. The dotted line represents the baseline level, corresponding to the closure of the unit conductance. Currents were filtered at 100 Hz.

and the residual conductances of the closed states, were not examined in detail.

In the light of results obtained by liposome swelling indicating that reduction of disulfide bonds was necessary to fully open pores within the chlamydial outer membrane, we attempted to oxidize our fully open MOMP channels and observe any effects on channel behavior. However, in vitro oxidation of bilayer-incorporated MOMP with hydrogen peroxide, oxidized glutathione, or Cu^{2+}-phenanthroline had no observable effect on any channel properties.

Ion selectivity and permeability of MOMP channels. The relative anion versus cation permeability of reconstituted MOMP channels was calculated from reversal potentials (see Materials and Methods) measured in 250 mM KCl *cis* versus 50 mM KCl *trans*. A reversal potential of $+10.0 \pm 4$ mV (mean ± SD, $n = 4$) indicated that the channel was weakly anion selective: $P_{Cl}/P_K = 2.0 \pm 0.84$ (mean ± SD, $n = 4$). The channels were also permeable to ATP. In the presence of 100 mM Na-ATP *cis* versus 10 mM Na-ATP *trans*, a reversal potential of -11 ± 1.9 mV (mean ± SD, $n = 10$) was obtained, with a unit conductance of approximately 80 pS. The ionic activity ratio of 100 mM Na-ATP to 10 mM Na-ATP, measured by using the Na^+ ionophore gramicidin (11), was 4.5. This corresponds to a theoretical (Nernstian) reversal potential of −39 mV for a solely Na^+ selecting channel and +10 mV for a solely ATP^{4-} selecting channel; hence, a reversal potential of −11 mV suggests that there is substantial ATP transport.

DISCUSSION

In the search for an effective antichlamydial vaccine, efforts have mainly concentrated on the proteins of the outer membrane. These proteins are likely to be the key virulence determinants of *Chlamydia*, involved in attachment, internalization, and the prevention of phagolysosomal fusion, and are an obvious starting point for vaccine development. Primarily, research has focused on the MOMP which is the predominant constituent of chlamydial outer membranes, comprising approximately 60% of the protein mass. The MOMP remains the only chlamydial protein shown unequivocally to be surface exposed, and antibodies specific to the protein have been shown to be protective (2, 28, 37). For these reasons, MOMP remains the primary candidate for a chlamydial vaccine.

Problems purifying native MOMP or obtaining recombinant MOMP in a native form have hampered the study of structure-function relationships for this protein. Despite this, MOMP has been implicated in many aspects of chlamydial pathogenesis (3, 12, 34–36). Perhaps one of the most convincing, and widely accepted, proposed functions is that of a "chlamydial porin." Using the technique of liposome swelling, Bavoil et al. (3) demonstrated that outer membrane complexes of *C. trachomatis* contained water-filled pores with an exclusion limit of 850 to 2250 Da. Due to its abundance in these outer membrane preparations, it was postulated that MOMP, as a chlamydial porin, was responsible. This hypothesis is amply supported by both the structural and functional results presented in this report.

The protocol used to purify MOMP for structural studies could not be employed during the purification of MOMP for reconstitution experiments due to the destabilizing effects of SDS on the bilayer. Unfortunately, dialysis to remove SDS from hydroxyapatite-purified fractions was not feasible because of the prohibitively small amounts of protein involved. However, both methods, solubilization in OG-DTT and purification by hydroxyapatite chromatography, resulted in the formation of SDS-resistant MOMP oligomers typical of classical gram-negative porin proteins. Structural analysis by CD indicated that the secondary structure of MOMP was mainly β sheet (62%), similar to the high β-sheet content of bacterial porins previously characterized by X-ray crystallography (29). The relatively high proportion of random coil, calculated from the MOMP spectrum, may be attributed to its four surface-exposed variable segments (1, 16, 41).

At a functional level, the insertion of MOMP into planar lipid bilayers seemed to be autocatalytic, resembling that seen with other porins (40). It is possible that the correct insertion of one channel assisted the correct orientation of other channel proteins, thereby accelerating subsequent insertion. The preference shown by MOMP for a particular bilayer orientation was demonstrated by the asymmetric response of the channel to holding potentials of opposite polarity. Presumably this asymmetry of behavior, seen in other porins, has a structural basis (9, 23). The modification of channel amplitude and open/closed transitions by the neutralizing MOMP-specific MAb A11 was of particular significance, for it not only confirmed that MOMP was the channel-forming protein but also reinforced our belief that MOMP is arranged as an oligomer in the bilayer, since A11 recognizes only oligomeric MOMP upon Western blotting (4, 21). This posed the question, What is the minimal conducting unit of the MOMP channel? From the

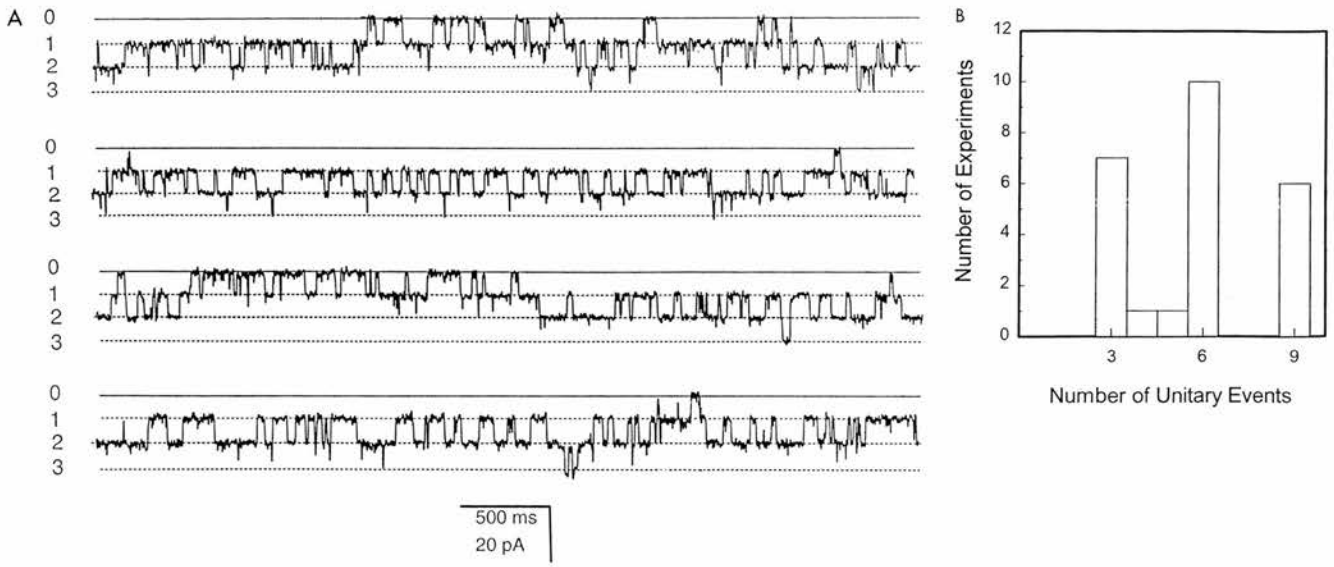

FIG. 6. Analysis of the minimal conducting unit of the MOMP channel. (A) Contiguous 40-s recording of MOMP activity in a bilayer bathed in 50 mM KCl–10 mM Tris-HCl (pH 7.4) (holding potential −70 mV, filtered at 200 Hz). The closed level of the channel (0) and three unit open levels (1, 2, and 3) are indicated. (B) Maximum number of unit open levels counted in 25 individual reconstitution experiments.

results summarized in Fig. 6, we suggest that each MOMP molecule inserts into the bilayer as a trimer to give rise to three pores through the membrane, and that switching to holding potentials of ±60 mV and above promotes the opening of all three pores together. Thus, two MOMP trimers could give rise to up to six pores with six unit currents. Voltage-dependent closure at maintained high holding potentials meant that within a few seconds, only one or two protochannels in each trimer were seen to be open at any one time. This type of multibarrel behavior is common in porins (25), and a similar phenomenon has been observed, and analyzed in detail, in eukaryotic channel proteins (e.g., references 7 and 15).

What ions and metabolites would be translocated across the chlamydial outer membrane by a MOMP porin? Previously, chlamydiae were thought to require host-derived nucleoside triphosphates as an energy source and precursor of RNA synthesis, prompting us to look at their transfer across the bilayer. The reversal potential of −11 mV observed in asymmetric concentrations of Na-ATP corresponded to the passage of substantial amounts of ATP across the bilayer. If the MOMP channel were Na^+ selective, allowing only the passage of Na^+, we would theoretically expect to see a reversal potential of −39 mV; alternatively, if the channel allowed the passage of only ATP^{4-} across the bilayer, a reversal potential of +10 mV would be observed. Therefore, a reversal potential of −11 mV demonstrates the passage of both Na^+ and ATP^{4-} through the MOMP channel. The identification of a route by which chlamydiae can take advantage of host nucleoside triphosphates and other nutrients may also explain why antibodies specific to MOMP neutralize infection. With the identification of genes encoding ATP biosynthetic pathways by the *Chlamydia* Genome Sequencing Project (32), it now appears unlikely that chlamydiae derive all of their ATP from the host cell.

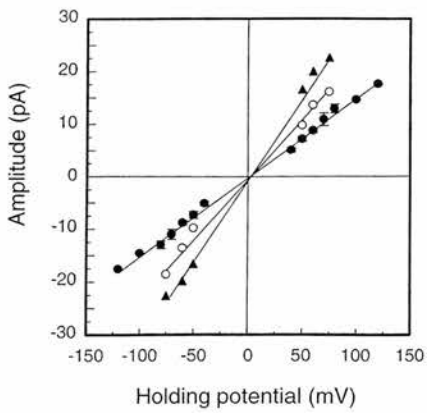

FIG. 7. Current-voltage relationship of MOMP channels in symmetrical concentrations of KCl (each buffered with 10 mM Tris-HCl [pH 7.4]). KCl concentrations are 300 mM (▲), 150 mM (○), and 50 mM (●). Error bars represent ± standard error of the mean for four independent experiments.

In the EB, outer membrane proteins are disulfide bond cross-linked, rendering the outer membrane largely impermeable. The transition into the intracellular RB is coupled with a reduction of outer membrane disulfide bonds, increasing membrane permeability. It is clear that MOMP's pore-forming activity would be primarily utilized at the RB stage of the chlamydial life cycle. Bavoil et al. (3) proposed that the reduction of outer membrane disulfide bonds opened chlamydial pores, allowing the uptake of ATP and other nutrients. Evidence supporting this hypothesis included activation of the pores by treatment with DTT and the blocking of reoxidation with iodoacetamide. Chemical modification was not required to maintain functional open channels from our OG-DTT-solubilized MOMP. We also noted that we could not reoxidize the putative free SH groups of bilayer-incorporated MOMP to close or inactivate the channels (mimicking the EB-to-RB transition). However, the reoxidation of disulfide bonds in the correct pairings to close the channel may require the presence of other proteins, such as the cysteine-rich proteins absent from the OG-DTT-solubilized sample.

In this study, we have shown that MOMP functions as a porin and have demonstrated a possible physiological function with the passage of nucleotide triphosphates through the channel. A particularly significant aspect of the work is that it provides a mea-

surable criterion by which to assess recombinant MOMP for native function. Indeed, we have recently reconstituted a recombinant MOMP into the planar lipid bilayer resulting in the formation of ion channels with properties identical to those of the native protein (39). These results have broad implications on the search for an effective chlamydial vaccine.

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